Epigenetic modulation of Doxcycline controlled transgene expression in cells and mice

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Contents

Summary

The tetracycline (Tet) regulation system is being widely utilized for conditional transgenesis in cells of various species because of its tight on/off regulation, lack of pleiotropic effects, high inducibility and fast response times. Although the system has been successfully used in mammalian cells, the application in mice is not as straight forward. Published examples show variations in the performance of the Tet system in mice: while some applications report about good expression and regulation，others showed methylation of promoter sequences which was linked to transcriptional silencing of the cassette. However, these studies differ in the selected chromosomal integration sites of the Tet cassettes, the design of the cassette, the tissue investigated as well as the nature of the read-out. This hampers the direct comparison and the improvement of the system.

The aim of this study was to characterize the performance of Tet cassettes located in a defined chromosomal environment in embryonic stem (ES) cells and mice and to explore strategies to overcome the silencing and heterogeneity of Tet induced transgene expression.

Three chromosomal loci in the mouse genome, Rosa26, COL1A1 and Tigre loci were evaluated for doxycycline induced expression. Although elevated expression levels have been observed in COL1A1 and Tigre loci in es cells compared to the expression in the Rosa26 locus, neither of them was capable of supporting expression of Tet cassettes upon differentiation, indicating that in all these sites expression is compromised. Further characterization indicated the involvement of DNA methylation. It was investigated if chromosomal shielding elements such as the chicken chromatin insulator cHS4 can prevent silencing of the Tet cassettes and stabilize the expression. Indeed, the introduction of cHS4 insulators into the Tet cassette resulted in partial rescue of expression in ES cells that was stable even after differentiation of the cells.

Furthermore, within this study, a novel strategy was designed to reactive the silenced Tet promoter in an active and targeted way. For this purpose, the catalytic domain from the ten eleven translocation methylcytosine dioxygenase 1 (TET1) was employed. This protein has been previously shown to induce the demethylation of methylated CpGs. To specifically recruit the catalytic active domain of the TET1 to the Tet promoter a tripartite fusion protein was designed

comprising the dioxygenase catalytic domain, the Tet promoter binding domain as well as the VP16 transactivating domain. This fusion protein was evaluated with respect to activate the Tet promoter. After expressing the fusion gene in ES cells, a significant increase of the transgene expression was observed which was dependent on the inducer doxycycline. This suggests that silencing of the Tet promoter can be overcome. Importantly, application of the expression vector in transgenic mice by hydrodynamic tail vein injection showed that the silenced Tet cassettes could even be reactivated in mice. In conclusion, this novel strategy to overcome silencing of the Tet promoter represents a promising approach to reactivate epigenetically silenced Tet promoter *in vitro* and *in vivo*.

Zusammenfassung/ kurzfassung

Die Tetracycline (Tet) regulierten systeme, unter Verwendung von Doxycycline induzierten Promotoren, werden häufig verwendet um eine konditionale Genexpression transduzierter Gene in Zellen verschiedener Spezies zu generieren, da diese Systeme eine besonders strikte Genregulation ermöglichen (an/aus), keine pleiotropen Effekte, sowie eine hohe Induzierbarkeit und kurze Antwortzeiten aufweisen.

Obwohl diese Systeme erfolgreich in Säugerzellen angewendet werden konnten, ist die Anwendung im Mausmodell nicht so geradlinig. Verschiedenste Publikationen zeigen Variationen im Verhalten des Tet-Systems im Mausmodell: einerseits wurde gezeigt, dass eine gute Expression und Regulation erreicht werden kann; andererseits wurde jedoch berichtet, dass die eingebrachten Kassetten epigenetisch „gesilenced“ werden, was auf die beobachtete Methylierung der Promotorsequenzen zurückgeführt werden konnte. Interessanterweise unterscheiden sich jedoch in all diesen studien die chromosomalen Integrationsorte, das Design der Kassetten, die verwendeten Gewebe als auch die Art der Auswertung.

Das Ziel dieser Arbeit bestand darin das Verhalten der Tet-Kassetten in embryonalen Stamzellen (ES-zellen) sowie in Mäusen zu charakterisieren und Strategien zu entwickeln das „silencing“ dieser Doxycycline-induzierbaren Promotoren zu verhindern, um somit eine homogenere und stabilere Genexpression zu erhalten.

Die erste Strategie verfolgte die Integration der Doxycycline-kontrollierten Kassetten in drei definierten chromosomalen Abschnitten (Rosa26, COL1A1 und Tigre Lokus) und die Untersuchung der Regulierbarkeit und Expression des Transgens. Obwohl vergleichsweise erhöhte Expressionslevel im COL1A1 und im Tigre Lokus in ES-Zellen gefunden wurden, konnte keiner der drei Integrationsorte die Expression der Tet-Kassette auch nach Zelldifferenzierung unterstützen. Nähere Untersuchungen zeigten, das DNA-Methylierung hier eine Rolle spielte.

Aus diesem Grund befasste sich die zweite Strategie mit der Modifikation der „targeting“-Kassette. Hierfür wurde der Doxycyclin-induzierte Promoter von dem „chicken HS4 insulator“ (cHS4) umrahmt. Durch die Anwesenheit des cHS4 konnte die Expression der

targeting- Kassetten erhöht und stabilisiert werden. Zudem ermöglichte die Integration des cHS4 eine teilweise erhaltene Expression des Transgens auch nach Zelldifferenzierung.

Des Weiteren wurde eine dritte Strategie entwickelt, welche die Reaktivierung bereits „gesilencter" Promotoren ermöglichen sollte. Für diesen Zweck wurde die aktive katalytische Domäne der „ten-eleven translocation methylcytosine dioxygenase 1" (TET1) mit dem reversen Tetrazyklin Transaktivator rtTA, ein Tetrazyklin kontrolliertes Transaktivator Protein, fusioniert. Das Protein TET1 wurde zuvor beschrieben eine Demethylierung von methylierten CpGs zu induzieren. Aufgrund dieses Fusionsproteins kann die TET1 katalytische Domäne direkt und Doxycycline-abhängig an den Tet-Promoter rekrutiert werden, da der rtTA nur in der Anwesenheit von Doxycycline an die Operator-Sequenzen des Tet-Promoters bindet. Somit sollte der Tet-Promoter induzierbar demethyliert und reaktiviert werden können.

Nachdem das Fusionsprotein transient in „getargeten" ES-Zellen exprimiert wurde, konnte eine signifikant erhöhte Expression der Reportergene beobachtet werden, welche abhängig vom Indikator Doxycycline war. Diese deutet darauf hin, dass ein „silencing" des Tet-Promotors überwunden werden kann. Beeindruckend, auch die Anwendung dieses Expressionvektors im Mausmodell ermöglichte eine Reaktivierung „gesilenc'ter" Transgenexpression. Hierzu wurde die Genexpressionskassette über hydrodynamische Injektion in zwei unterschiedlichen transgenen Tierlinien eingebracht und seine Wirkung untersucht. In beiden Fällen führte die transiente Integration zu einem signifikanten Anstieg des Reportergens.

Zusammenfassend zeigt sich diese neuartige Strategie als sehr vielversprechend das „silencing" des Tet-Promotors *in vitro* und *in vivo* zu überwinden und zeigt sich ebenfalls erfolgreich in der Reaktivierung zuvor „gesilencter" Tet-Promotoren.

1. INTRODUCTION

1.1 TRANSGENSIS

Genetically modified animal models are powerful tools in experimental and applied biology. The expression of heterologous genes (transgenes) in host allows the researchers e.g. to study the functionality of genes *in vivo*, to establish pathologic models for human disease and also enables the production of pharmaceutical proteins.

For the generation of transgenic animals, zygote modification and ES cell mediated transgenesis are broadly used. Via these methods the transgenes are stably integrated into the genome of the host and support long-term expression. There are two patterns of the insertion of transgenes into the host genome: random integration and targeted integration.

1.1.1 Random transgene integration and problems

Since Gordon and his colleagues established the first transgenic mouse by pronuclear injection (PI) of a foreign DNA sequence into zygotes 30 years ago (Gordon, Scangos, Plotkin, Barbosa, & Ruddle, 1980), transgenesis was attracting increasing attention of scientists. Accordingly, this led to the fast progress in developing new technologies to transfer foreign DNA to recipients like DNA microinjection (Bishop & Smith, 1989) or ES cell modification (Bartlett, 2010). However, these methods are hampered by the uncontrollability of transgene expression. The first drawback that might influence transgene expression is unpredictable copy number (Ashe et al., 2008) since multiple-copy integration frequently led to tandem transgene arrays.These would induce so-called repeat-induced gene silencing (RIGS) caused by heterochromatin formation (Garrick, Fiering, Martin, & Whitelaw, 1998). Moreover, random insertion of transgene could also pose the transgene susceptible to the position effect which is induced by the neighbouring elements like ubiquitous chromatin opening elements (UCOEs), Locus control regions (LCRs) and Scaffold/matrix attachment regions (S/MARs) (Allshire, Javerzat, Redhead, & Cranston, 1994; Argyros et al., 2011; Yao et al., 2003; F. Zhang et al., 2010a).Vice versa, the transgenes could

also exert impacts on endogenous gene expression. In order to avoid these drawbacks and achieve predictable and reliable transgene expression, strategies for targeted transgenesis in a defined genomic locus have been explored and utilized in generating transgenic cell lines and animals.

1.1.2 Targeted transgenesis

As discussed in the previous chapter, random integration results in unexpected interactions between transgenes and the host genome. This limits the reliability of transgene expression. Therefore, targeted integration was preferred by researchers later on (Papapetrou et al., 2011).
To achieve targeted genomic editing, homologous recombination (HR) is widely used. HR is providing an exchange of nucleotide sequences between two similar or identical molecules of the DNA. For this purpose, double-strand breaks (DSBs) are always performed together with HR to fulfill high HR efficiency. In order to actualize site-specific DSBs, three major engineered DNA-binding proteins have been exploited to date: zinc finger (ZF) nucleases based on eukaryotic transcription factors (Maeder et al., 2008), transcription activator-like effector nucleases (TALENs) derived from *Xanthomonas* bacteria (Hillen & Berens, 1994; Maeder et al., 2008), and most recently the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system CRISPR (Cheng et al., 2013; Cong et al., 2013; Fu et al., 2013; Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Hsu, Lander, & Zhang, 2014; Sapranauskas et al., 2011; Xue et al., 2014).

1.1.2.1 RMCE

Site specific recombinase mediate cassette exchange (RMCE) is a powerful tool for targeted transgenesis which have been successfully utilized to achieve predictable gene expression in cell culture and transgenic animals (Kues et al., 2006). Two RMCE systems are widely exploited: (i) Cre/loxP mediated cassette exchange and (ii) FLP/FRT mediated cassette exchange. In these systems the Cre and FLP are recombinases which could recombine a pair of short target sequences called the loxP sequences or FRT sequences. In this way cassette exchanges are

achieved between the same FRT/loxP sites (Nehlsen, da Gama-Norton, Schucht, Hauser, & Wirth, 2011) .

To establish a transgenic mouse by RMCE all the embryonic stem (ES) cells need to be tagged, i.e. the insertion of two different FRT sites into the target chromosal locus (eg. Rosa26) by homologous recombination (HR) (An et al., 2012; Sandhu et al., 2011). After that the vector that comprises the gene of interest (GOI) flanked with the same FRT sites in a proper orientation has to be transfected to the cells together with the FLP recombinase (Baer & Bode, 2001). In this way the GOI will integrate into the previously modified genomic locus.

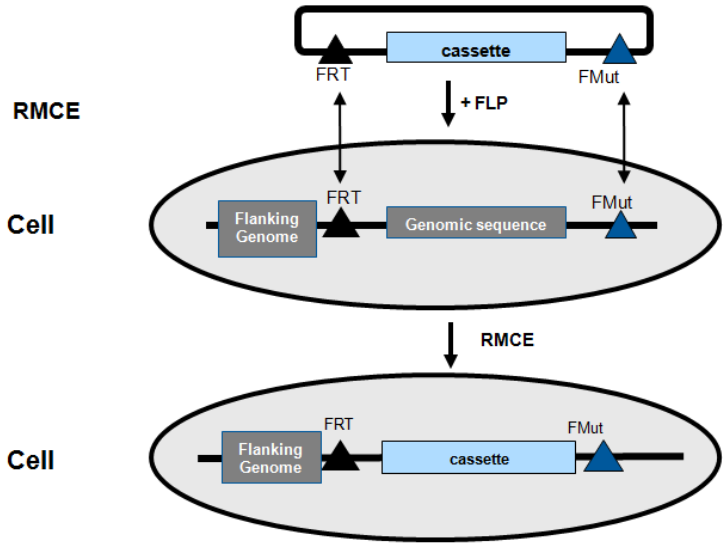


Figure 1. Principle of RMCE. A plasmid comprising the FRT sites flanking the GOI (gene of interest) was co-transfected with FLP recombinase, which results in the cassette exchange between the same FRT sites and FRT mutant (FMut) sites in the cells.

1.1.3 Characterized chromosomal loci in the mouse genome

A huge number of potential integration sites exist in the mouse genome, but only few are characterized for transgene expression. Importantly, the performances of the cassettes in the different loci are not predictable and have to be tested experimentally. A number of genomic loci have been used to integrate transgenes up to now and the most important loci relevant for this thesis are described in the following briefly.

1.1.3.1 Rosa26 locus

Rosa26 locus was first identified by Soriano and his coworkers in mouse embryonic stem (ES) cells in 1991. Its virtues of ubiquitous expression in all tissues and frequently observed gene targeting events in mouse ES cells inspired people to introduce transgenes into this locus. Afterwards, more than hundred mouse lines expressing various transgenes in this locus were established successfully using different strategies (Casola, 2010).

Rosa26 locus is located on chromosome 6 and transcribes three mRNAs with unclear function. Disruption of these transcripts does not lead to any observed negative effects on animal's life (Zambrowicz et al., 1997). In respect to the above mentioned advantages Rosa26 locus became one of the most popular loci applied for targeted transgene expression.

1.1.3.2 COL1A1 locus

The COL1A1 locus is located on chromosome 17 and encodes type I collagen which is expressed in most connective tissues. COL1A1 was found to be a good candidate for targeting because it is highly expressed in fibroblast and ES cells. Because the transgenes were always integrated into the 3' region of CoLlAI, no impair of CoL1A1 expression has been observed up to now. Also doxcycline-induced transgene expression cassettes have been introduced into this locus. Of note, regulated expression was reported both *in vitro* and *in vivo* (Krestel et al., 2004; Pichlmair et al., 2012; Yu et al., 2013)(Beard, Hochedlinger, Plath, Wutz, & Jaenisch, 2006a; Prockop & Kivirikko, 1995; Stadtfeld, Maherali, Borkent, & Hochedlinger, 2010).

This locus is well characterized and depicted in Figure S1. The targetable ES cell line is tagged with one FRT site in COL1A1 and was established by Beard et al. (Beard, Hochedlinger, Plath, Wutz, & Jaenisch, 2006b). The targeting of this locus includes a complete integration of the plasmid (Figure S1b). The whole bacteria backbone was integrated to the endogenous Collagen locus through FRT wt sites by recombinase mediated cassette exchange (RMCE). This targeting strategy is different from that for the Rosa26 locus (Fig S1a).

1.1.3.3 Tigre locus

The Tigre locus, referring tightly regulated locus, is a recently discovered targeting candidate. It was identified in 2008 by Zeng and his coworkers. The Tigre locus is located on chromosome 9 encoding for the AB124611 gene and Carm 1 gene. Furthermore, no lethality has been observed by transgene insertion (Zeng et al., 2008). Importantly, the Tigre locus was screened specifically for regulated transgene expression using Tet cassette, which made it different from the previously mentioned loci (Zeng et al., 2008).

1.1.3.4 Other loci

Besides the characterized genomic loci highlighted above, there are also other loci: Hipp11 (H11) is named by Hippenmeyer et al. and is located on chromosome 11; H2-Tw3 is located on chromosome 17 and encodes a non-classical major histocompatibility complex (MHC) antigen (Olivares-Villagómez et al., 2011) and the Hprt locus is located on the X chromosome encoding an enzyme required for the salvage pathway of purine biosynthesis. All of these loci have been reported to shield transgene expression without disturbing endogenous gene expression (Palais et al., 2009).

1.1.4 Inducible promoters for controlled transgene expression

As the name implies, inducible promoters exhibited the capacity of controlled transcription activity induced by the presence or absence of inducers (biotic or abiotic factors). This property makes inducible promoters useful tools in genetic engineering as the transgene expression could be temporally and spatially controlled. Synthetic inducible promoters as well as natural inducible promoter exist. Currently, the Tet promoter is the most commonly and widely used synthetic inducible promoter, which is also used in this thesis.

1.1.4.1 Advantages of inducible expression of transgenes

An uncontrolled expression of a transgene might lead to disability of the transgenic animal or embryonic lethality mediated by overexpression of potentially toxic genes. This might hamper further studies and lead to the silencing of transgenes.

Benefiting from the regulated expression systems it is possible to switch on the transgene expression in the target cells at favored time point and thereby rule out the disadvantages brought by constant promoters (Belteki et al., 2005). Furthermore, regulated expression systems allow the induction of different expression phenotypes in the same mouse, thereby allowing to analyze the kinetics (Haenebalcke et al., 2013).

1.1.4.2 The tetracycline inducible promoter

The tetracycline (Tet) inducible promoter, which can be induced by tetracycline (or its derivate doxycyline), is a synthetic promoter that consists of several (usually seven) tandem repeats of the 19 bp bacterial Tet-operator (Tet-O) sequence separated by spacer sequences combined with a minimal eukaryotic promoter (normally derived from the Cytomegalovirus promoter, designated as CMV mini).

The Tet-inducible systems can be subclassified into the Tet-on system and the Tet-off systems, which differ in the used transactivators. In the Tet-On system, reverse tetracycline-controlled transactivator protein (rtTA) is disassociated from the Tet–O sequences in the presence of doxycline. And the absence of doxycycline induces inactivated transgene expression by binding of rtTA to the Tet-O sequences (Figure 2). In contrast to that, in the Tet-Off system, the situation is opposite. The transactivator protein (tTA) binds to the Tet-O in absence of doxycycline and activates the transgene expression, while the transgene expression is inactivated in the presence of doxycycline. (Loew, Heinz, Hampf, Bujard, & Gossen, 2010)

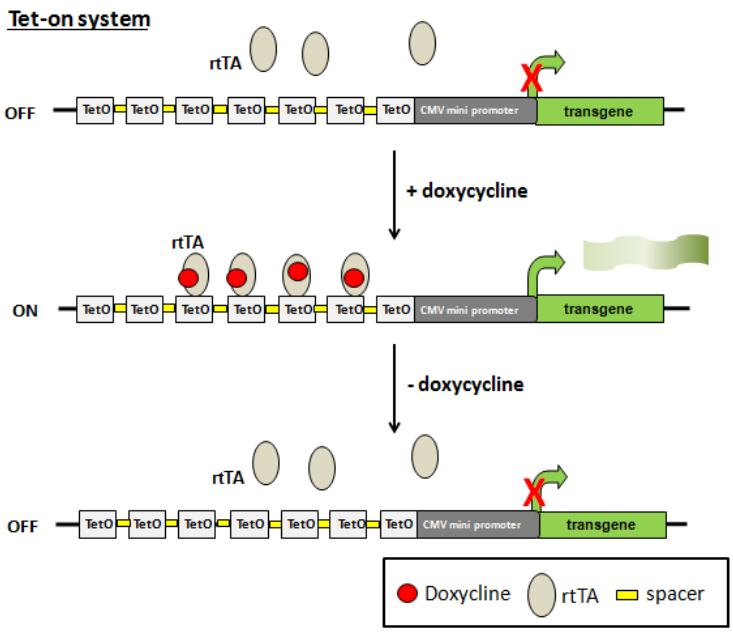


Figure 2: Principle of Tet-On inducible system. The classical Tet promoter is composed of a minimal CMV promoter which has only the TATA box for initiation of transcription, 7 Tet operation regions for rtTA recruiment and spacers in between of the Tet operators .In the Tet-On system, the reverse tetracycline-controlled transactivator protein (rtTA) is disassociated from the Tet operator sequence in the absence of doxycycline and induces inactivated transgene in presence of doxycycline.

1.2 EPIGENETIC MODULATION OF TRANSGENE EXPRESSION

1.2.1 Epigenetic modifications

Interestingly, the entire human population irrespective of genders, races or age periods shares 99.9% identical sequences. However, we still differ from each other in a thousand ways. Researchers are inextricably bogged down in the torturing of the question "what is the other recipe beyond DNA". In 1942 the word "epigenetics" was first coined and defined by Conrad Waddington. From the word "epi" referring "above" literally, one could grip the point of this word with consummate ease as "something happened above genetics", which is quite close to its official definition "causal interactions between genes and their products which bring the phenotype into being" (Livingstone, 2012; Sui, Price, Li, & Chen, 2012).

Lots of publications were followed afterwards on the subject of epigenetics, which proved that the influence of epigenetics on gene expression is in a controlled and selective manner and even inheritable. Several epigenetic mechanisms interfering gene expression have been discovered up to now. DNA methylation and chromatin remodeling play central roles in epigenetic modification (Pai, Bell, Marioni, Pritchard, & Gilad, 2011; Ptashne, 2007), as well as microRNAs and sRNAs mediated modifications (Mattick, Amaral, Dinger, Mercer, & Mehler, 2009; Meyer, 2013), RNA transcripts and their encoded proteins induced transcription boost or abate (Howden et al., 2013) and non-DNA inheritance systems conducted by structure (Sapp, 1991).

1.2.1.1 DNA methylation

In DNA methylation, CpGs play a critical role. CpG sites are the DNA regions with a significant higher amount of binucleotides, which consist of a cytosine nucleotide next to a guanine nucleotide and linked by a single phosphate in the linear sequence. These sites are prone to DNA methylation (Jabbari & Bernardi, 2004; Nan, Meehan, & Bird, 1993). When a methyl group is deprived from the substrates S-adenosyl-L-methionine (SAM) and then adds to the 5' position of cytosine, a 5-methylcytosine in CpG site is formed. This process is mediated by DNA methyl-transferases (DNMTs) (Ashe et al., 2008; Jaenisch & Bird, 2003) (Figure 3).

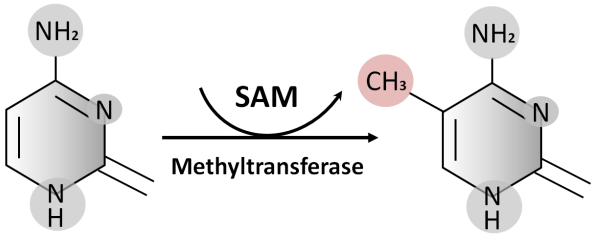


Figure 3: Mechanism of DNA methylation at cytosines. DNA methylation reactions are catalyzed by DNA methyltransferase (DNMT) in the way of depriving a methyl group from S-adenosyl methionine (SAM) to the 5' position of cytosine and form 5-methylcytosine.

Condensed methylated regions of CpG sites are tending to be located near a transcription start sites, within gene promoter regions and enhancer regions (Dahl, Grønbæk, & Guldberg, 2011; Ndlovu, Denis, & Fuks, 2011). Condensed methylated CpG areas in the genome tend to be silenced or at least are less transcriptionally active, especially when they are located in the

promoter regions of the genes. Methylation patterns can also be inherited from parents into the zygote, which makes the methylation codes pass on from generation to generation (Choy et al., 2010). The proposed mechanism of gene regulation is the competition of binding between transcription factors and methylation recruited co-repressors to the DNA. Once methylcytosines are formed, a complex composed of methyl CpG binding protein 2 (MeCP2) (Nan et al., 1993), Histone deacetylase (HDAC) and methyl binding proteins (MBPs) 1 – 4 (Meehan, Lewis, McKay, Kleiner, & Bird, 1989; Nan et al., 1993) are speedily summoned and occupy the methylated region. This impedes the binding of the transcription factors such as transcription initiation factor (TAF), TATA-binding protein (TBP) and RNA-polymerase 2. As long as the transcription factors can not access and have no chance to bind the DNA, transcription can not be initialized and the expression of gene will be switched off or silenced (Newell-Price, Clark, & King, n.d.) (Figure 4).

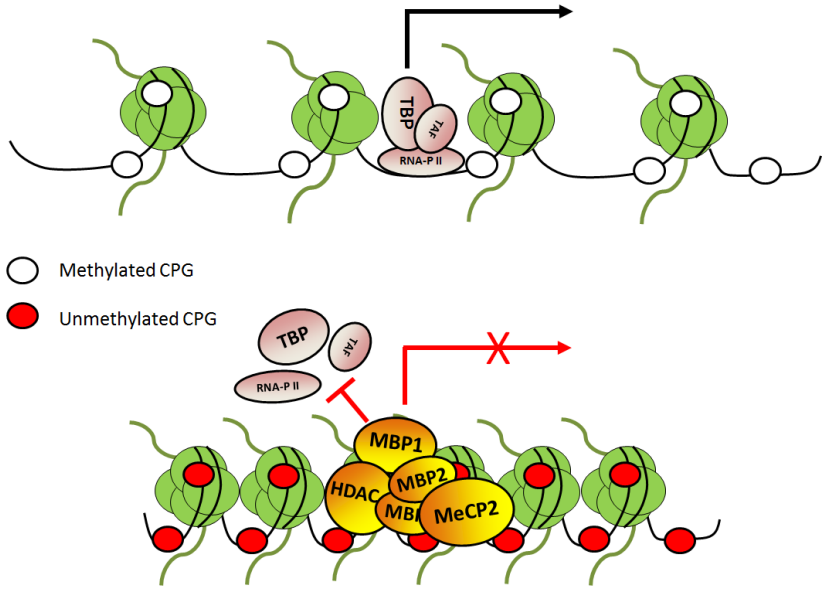


Figure 4: Mechanism of DNA methylation induced gene suppression. Unmethylated DNA could form a loose loop, which allows transcription factors such as transcription initiation factor (TAF), TATA-binding protein (TBP), and RNA-polymerase 2 to bind to the DNA. This initiates the transcription. Once a certain level of cytosines in CpG sites is achieved, the chromatin can not maintain at a loose status. Instead, MeCP2, HDAC and MBPs will be fast recruited to the region and compact the genome to a status of inaccessibility. As a result other transcription factors can not get in touch with DNA and gene expression is repressed (Gupta et al., 2010).

1.2.1.2 Passive and active DNA demethylation

Importantly, DNA methylation is reversible or can even be impeded. Methyl groups from nucleotides can be removed by a process called DNA demethylation. The process of DNA demethylation can be divided into two types: passive DNA demethylation and active DNA demethylation.

Passive DNA demethylation and DNA methyl-transferase (DNMT) inhibitors

The passive DNA demethylation is unnatural and mediated by DNMT inhibitors when replications are taken place and new DNA strands are synthesised. 5-azacytidine (Aza) is a chemical analogue of the cytosine nucleoside which induces the inhibition of DNMTs through its incorporation into DNA and RNA (Pedram et al., 2006; Stresemann & Lyko, 2008). When DNMTs recognize azacytosine-guanine dinucleotides, methylation process will be initiated by a nucleophilic attack which establish a covalent bond between the enzyme and the carbon-6 atom of the cytosine (Lübbert, 2000). Unlike what will happen to the natural cytosine, this bond formed between Azacytosine and the enzyme could not be resolved by beta-elimination through the carbon-5 atom, because carbon-5 in Azacytosine has been replaced by nitrogen. This mechanism enables an inhibition of DNMTs by treatment with Aza. DNMTS can not function appropriately and DNA methylation process is blocked. Importantly, in consideration of the fact that this blocking effect could only be exerted when azacytosine incorporated into the genome, azacytosine induced DNA methylation is restricted to proliferating cells (Stresemann & Lyko, 2008).

Decitabine is a hypomethylating agent. It is another chemical analogue of the cytosine (Kantarjian et al., 2006; Stresemann & Lyko, 2008). Decitabine hypomethylates DNA by inhibiting DNMTS in the same manner like Aza. In contrast to Aza, Decitabine can only incorporate into DNA chains and not into RNA chains

Active DNA demethylation and the role of TET proteins

The blocking of DNA methylation is an induced, non-natural process. Instead, active DNA demethylation mechanisms have been discovered in recent years as a natural demethylation process. E.g. zygote formation after fertilization which is entirely independent of neither DNA

replication nor any help from utilization of extra external chemicals displays a high reduction of methylation of 5' cytosines (X. Li et al., 2008). This indicates the presence of an active demethylation mechanism in these processes. For a long time the mechanism of active demethylaiton in mammalian cells was not clear. However, in 2009 a breakthrough was achieved in mammalian cells by identifying ten-eleven translocation methylcytosine dioxygenase 1 (TET1) protein as an important player in active demethylation of mammalian DNA (Guo, Su, Zhong, Ming, & Song, 2011a, 2011b; Wu & Zhang, 2011). Afterwards TET2 and TET3 were also discovered as the members of the TET family. They have been also discovered exhibiting 5mC hydroxylase activities (Ito et al., 2010). There are several highly conserved domains found in all TET proteins which have been demonstrated indispensable in their demethylation activities. This includes (i) a CXXC domain, also known as CpG-binding protein (CGBP), (ii) a CXXC-type zinc finger protein which is recruited almost exclusively to clustered CpG sites especially to the methylated ones, and (iii) a catalytic domain which is a Fe (II)- and 2-oxoglutarate (2OG)-dependent dioxygenase (Figure 5).

TET1 was initially identified in acute myeloid leukemia (AML) as a fusion partner of the histone H3 Lys 4 (H3K4) methyltransferase and mixed-lineage leukemia (MLL) in 2003 by Lorsbach and his colleagues (Lorsbach et al., 2003). In 2009, Zhang and his colleagues convincingly showed that human TET1 protein possesses enzymatic activity capable of hydroxylating 5-methylcytosine (5mC) to generate 5-hydroxymethylcytosine (5hmC) by oxidation of 5mC in an iron and α-ketoglutarate dependent manner. Of note, this conversion of 5mC to 5hmC has been proposed as the initial step of active DNA demethylation in mammals (Dahl et al., 2011; Dawlaty et al., 2011; Tahiliani et al., 2009; Williams et al., 2011; H. Zhang et al., 2010) which was later confirmed by various experimental systems (Guo et al., 2011a, 2011b; Wu & Zhang, 2011).

As 5mC has been considered long time to be a unique DNA modification in mammals, the discovery of 5hmC opens up the new field for regulation of DNA methylation. Researchers found that the levels of 5hmC are varying between different cell types and tissues. Most is found in the brain especially in neurons (Globisch et al., 2010; Gu et al., 2011; Kriaucionis & Heintz, 2009; Piccolo et al., 2013; C.-X. Song et al., 2011).

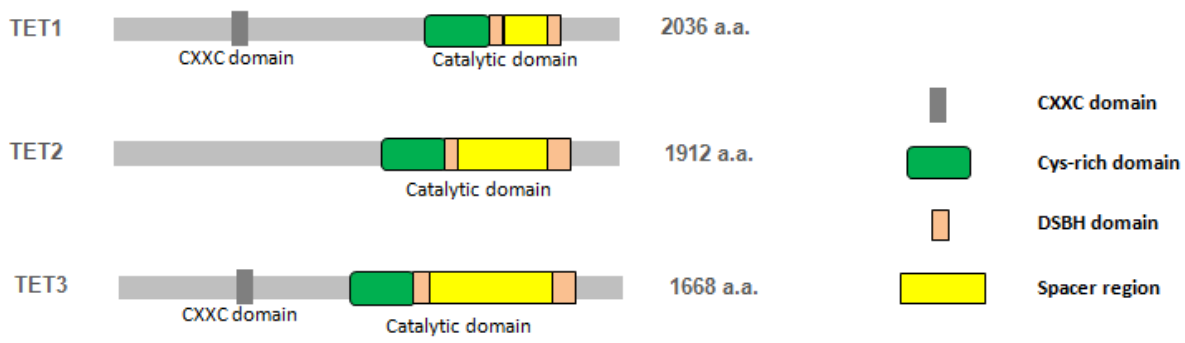


Figure 5: The conserved structures of TET protein family. CXXC-type zinc finger protein domain (CXXC domain) is responsible for the binding of TET proteins with CpG sites and the catalytic domain consists of a Cysteine rich domain, spacer and double strand βhelix domain which exhibits 2-oxoglutarate (2-OG)- and iron (II)-dependent dioxygenase activity. TET1 and TET3 comprise both domains while TET2 lacks CXXC domain for binding. Although all the TET proteins contain catalytic domains, which are composed of DSBH, Cys-rich domain and a spacer, slight differences in the size of each domain could still be observed which might explain the differences in functionality of TET family proteins.

He and his colleagues came up with a potential DNA demethylation process and showed in 2011 that TET proteins are capable oxidizing 5hmC to 5-formylcytosine (5fC) and further to 5-carboxylcytosine (5caC). These were detectable in genomic DNA of mouse ES cells. Importantly, when TET1 protein has been knocked down in mouse ES cells the levels of 5fC and 5caC dramatically shrinked. This might imply that 5hmc catalyzed by TET proteins is the primary step for the demethylation cascade (He et al., 2011; Ito et al., 2011) **(Figure 6).**

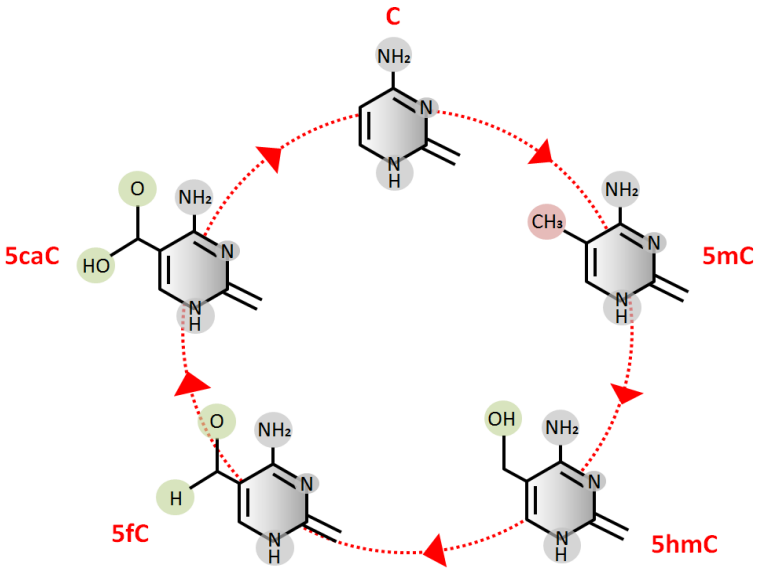


Figure 6: The step-wise cytosine demethylation mechanism. After the methyl group from S-adenosylmethionine (SAM) is transferred to the cytosine with the catalysis of DNMTs, Fe(II)/α-ketoglutarate (a-KG)-dependent dioxygenases, TET proteins will revert the methylated bases of the DNA into the catalytic site by a base-flipping mechanism. Afterwards, the active site Fe(II) is bound by the His-His-Asp residues in TET proteins and this concerts the working of a-KG and water. Oxygen is utilized by TET proteins as a substrate to catalyze oxidative decarboxylation of a-KG and produce enzyme-bound succinate, and a reactive high valent Fe(IV)-oxo intermediate which then reacts with 5mC, 5hmC and 5fC to generate 5hmC, 5fC and 5caC, with a net oxidative transfer of the single oxygen atom to the substrate which lead to the regeneration of the Fe(II) species.

1.2.1.3 Histone modifications

Besides the DNA methylation, histone modifications are considered to be important epigenetic modifications that play a crucial role in the gene regulation. Histone modifications always occur on long tailed H3 and H4 histones. A large number of different histone modifications have been reported. They comprise for example acetylation, methylation, SUMOylation, phosphorylation, citrullination and ubiquitination. They are considered play a crucial role and act in e.g. gene regulation, DNA repair and chromosome condensation (Gupta et al., 2010; Litt, Simpson, Recillas-Targa, Prioleau, & Felsenfeld, 2001; N. Song et al., 2011; Sui et al., 2012).

1.2.1.4 Choromosomal elements and their proposed epigenetic modulation

After a transgene is integrated into the recipients' chromosomes, partial or complete loss of transgene expression occurs over time upon passaging, freezing or differentiation which might be explained by the chromosal position effects (Ellis, 2005; D W Emery, Yannaki, Tubb, & Stamatoyannopoulos, 2000; David W Emery, 2011). Although some researchers claim the position effect could be avoided by screening of some endogenous loci or single copy insertion, silencing problem has not been thoroughly settled by either of these solutions (Persons, Hargrove, Allay, Hanawa, & Nienhuis, 2003).

However, as a mechanism for transgene silencing also spreading of the heterochromatin has been discussed. The spreading is frequently found in the genomes of advanced eukaryotes that silenced the transgene expression. Accordingly, spreading of heterochromatin might result in condensed chromatin and abolish the accessibility of transcription factors (Raab & Kamakaka, 2010; West, Gaszner, & Felsenfeld, 2002).

Chromatin insulators

Chromatin Insulators, first identified in Drosophila, are DNA sequences which act as genetic boundary to protect target gene from the impact of enhancers or silencers in neighboring domains, or prevent the spreading of the upstream heterochromatin by insertion in between neighboring domains and target genes (Burgess-Beusse et al., 2002; Recillas-Targa et al., 2002). Chromatin insulators have been found naturally in the genome of eukaryotes that could form functional boundaries between adjacent chromatin domains, which play critical roles in gene regulation and genomic architecture. Two classes of chromatin insulators have been discovered until now: the enhancer-blocking insulators and barrier insulators (Burgess-Beusse et al., 2002; West, Huang, Gaszner, Litt, & Felsenfeld, 2004; C. Zhang, Huys, Thibault, & Wilson, 2012).

The enhancer-blocking insulators are capable of interfering with enhancer–promoter interactions when placed between them. These act by recruiting nuclear protein CCCTC-binding factors (CTCF). Insulators themselves could form physical loop structures that are established through CTCF-mediated interactions, although this hypothesis could not account for all cases of enhancer blocking activity (Cuddapah et al., 2009; Guelen et al., 2008; Herold, Bartkuhn, & Renkawitz, 2012; Hou, Dale, & Dean, 2010; Tao Li et al., 2008; Tie Li, Lu, & Lu, 2004; Ling et al., 2006; Ohlsson, Bartkuhn, & Renkawitz, 2010; Rubio et al., 2008; Sanyal, Lajoie, Jain, & Dekker, 2012) **(Figure 7).**

The barrier insulators are protecting target genes against position-effect variegation (PEV)- a variegation caused by the silencing of a gene through its juxtaposition with heterochromatin (Carabana, Watanabe, Hao, & Krangel, 2011; Singer, Liu, & Cox, 2012).

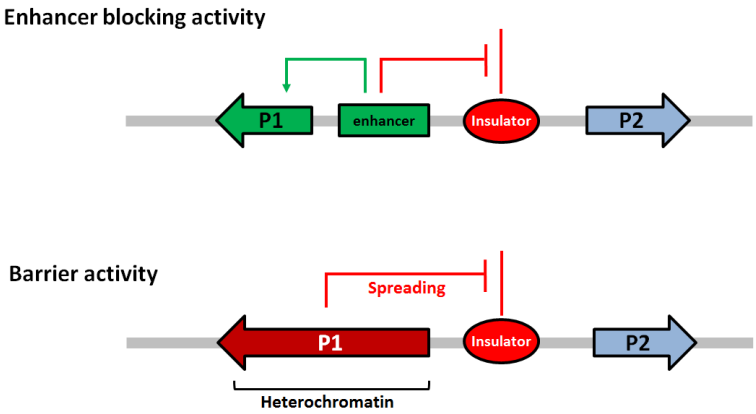


Figure 7. Schemes elucidate the mechanism of an enhancer blocking insulator and a barrier insulator. As shown in the upper part of the figure, the enhancer-blocking insulator is the DNA sequence which could efficiently blocks external enhancer's activity from promoter (P1) on the target promoter (P2) when placed between them. And for barrier activity, the insulator should be capable of recruiting a barrier complex to prevent the spreading of heterochromatic domains.

cHS4 insulator

The Chicken β-globin locus is located on chicken chromosome 1 downstream of the *folate receptor (FOLR1)* gene. It is a 1.2 kb DNase I-hypersensitive site (5'HS4) region located in between a ~16 kb long condensed chromatin domain that sits downstream of the *folate receptor (FR)* gene and a globin cluster and functions as an chromatin insulator (Chung, J H). Barrier function of 5'HS4 was naturally conceived (Burgess-Beusse et al., 2002). Furthermore, upstream stimulatory factor1/2 (USF1/USF2) has been characterized within the 1.2kb DNase I-hypersensitive site (5'HS4) region. USF1 and USF2 are responsible for recruiting enzyme complexes capable of modifying histones on adjacent region with 'activating' markers (Litt et al., 2001). Moreover, vascular endothelial zinc finger 1 which functions for protecting DNA against DNA methylation has been identified in 5'HS4 as well (Lachner, O'Carroll, Rea, Mechtler, & Jenuwein, 2001). Subsequent, a CCCTC-binding factor (CTCF) recruiting domain has also been identified in the 1.2 kb 5'HS4 sequence. CTCF was identified years ago to support the

stabilization of long-range interactions in the nucleus and to exhibit enhancer blocking activity. (Bartkuhn et al., 2009; Holohan et al., 2007; Kanduri et al., 2000; Ohlsson et al., 2010) (Figure 8). Plenty of publications followed up and showed that transgene expression could be protected from silencing by the insertion of single or two copies of 5'HS4 full length elements or simply by insertion of the core elements only (Bell, West, & Felsenfeld, 1999; Burgess-Beusse et al., 2002; Recillas-Targa et al., 2002).

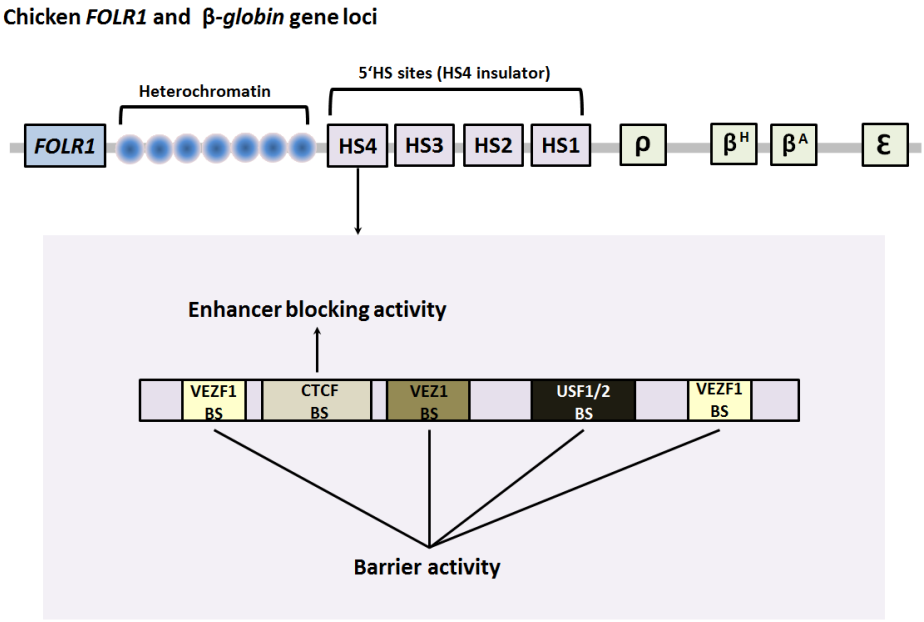


Figure 8: The chicken HS4 insulator. CHS4 insulator is the 1.2 kb cis-element fragment compromising a hypersensitive site 4 at the 5′ end of the chicken β-globin gene. This element exhibits both enhancer-blocking activity and barrier activity effects cross-species. It locates between the folate receptor 1 gene and the β-globin locus and protects the active β-globin gene domain against heterochromatin. On the one hand, a CTCF binding site located in the 5′ of CHS4 insulator will recruit CCCTC-binding factor (CTCF) to exert enhancer blocking activity. On the other hand, VEZF1, VEZ1 and USF1/2 binding sites are used for attracting proteins for barrier activity as vascular endothelial zinc finger 1 (VEZF1) which is essential for resisting DNA methylation, and upstream stimulatory factors (USFs) which are critical for histone modification.

1.2.2 Crosstalk between transgene and endogenous genes

The crosstalk of externally introduced and integrated transgenes into recipient's genome with the genomic environment has becoming a hot topic in recent years. Such a crosstalk could be

observed regardless of the copy number of integration and random or locus specific insertion (Berry, Hannenhalli, Leipzig, & Bushman, 2006; Meyer, 2013; Singer et al., 2012). For instance, the genomic context and chromatin structures flanking the integration sites could exert impacts on the integrated transgenes. It has been reported that silencing or heterogeneous expression of the transgene in cell clones was closely related to the endogenous genomic sequences (Cai & Shen, 2001; Ellis, 2005).

Vice versa, the transgenes could also influence the endogenous genes expression. This might affect genes within the flanking region of integration or even distant genes which directly or indirectly interact with transgene by the folding of the genome (David W Emery, 2011; Kang, Kwon, Lee, & Seo, 2013).

Impact of chromosomal integration sites on Doxycline induced cassettes

The doxycline inducible promoter (Tet-promoter) has been broadly utilized for establishing transgenic animal models and cell lines for its merit of controllability. During the last decades people achieved impressive success as numerous transgenic cell lines and animal models with tight regulated expression - controlled by Doxycline treatment - have been established. By expressing the transactivator under control of a tissue specific promoter, even spatial (tissue-specific) and temporal control of expression could be achieved (Beard et al., 2006b; Sandhu et al., 2011; Thesis Kruse, 2013). However, while regulated expression was reported on ES cell level, expression on the level of transgenic animals seems to be heterogenous and less predictable (Bao-Cutrona & Moral, 2009; Thesis Kruse, 2013). Previous results suggest that unexpected expression patterns of tetracycline-regulated transgenes in animals and cells (Bao-Cutrona & Moral, 2009) were observed with increasing frequency. In some studies employing the Tet system to the central nervous system (CNS), the reactivation of transgene was hardly achieved at high level (Krestel et al., 2004; S. Uchida et al., 2006). They explained the observation by a weak PTet/PTetbi activation in adult mice or PTet/PTetbi susceptibility to silencing which might be induced by DNA methylation (Kues et al., 2006). These observations lead the scientist increasingly to the questions why the transgene expression was not foreseeable and how the mechanisms could be controlled.

Several ES cell lines and mouse strains have been established in our lab applying Tet induced transgene expression. Surprisingly, heterogeneous expression patterns have been observed in all

ES cell lines and mouse strains which contained a single copy insertion of Tet cassette in Rosa26 locus, irrespective of the version of Tet promoter or the types of transgene. Methylation analysis on Tet promoter region from the existing ROSA GFP mice (Thesis Spencer, 2014) revealed 90-99% methylated CPGs in Tet promoter region isolated from mice or cell lines displaying low or no transgene expression. Interestingly, in those cells and mice the methylation level of Tet promoter decreased to 40-50% after Aza treatment. This indicates that DNA methylation might contribute to heterogeneous and low expression pattern of the Tet cassettes in the Rosa26 locus.

1.2 Aim

Although Tet cassettes are reliable inducible tools for many applications, the failures of Tet induced transgene expression have been observed in many integration sites. Since an inducible transgene expression is of interest for many researchers, it would be beneficial to overcome this challenge and establish a predictable and stable inducible transgene expression.

Therefore, overcoming the silencing of Tet cassettes *in vitro* and *in vivo* was the aim of this study.For this purpose, different approaches will be investigated to rescue the expression of the Tet cassette.

First of all, Tet cassette will be evaluated at different defined chromosomal integration sites (Rosa26 locus, COL1A1 locus and Tigre locus). Secondly, chromosomal modifiers like cHS4 insulator will be introduced to shield the Tet cassette and the expression profile of the modified Tet cassette will be investigated *in vitro* and *vivo*. Last but not least, active demethylation of Tet cassette will also be investigated in cells and in mice in different loci by TET1.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Equipments

Table 1. Equipments

Equipments	**Manufacturers**
Autoclave	BelimedDampf Sterilisator 6-6-6 HS1, FD
BLI system	Labotect
Gel Electrophoresis Chambers	Gibco BRL Horizontal Gel Electrophoresis Apparatus
Cell Counter	Guava EasyCount, Millipore
Cell culture incubators	Forma Scientific Water jacketed Incubator
	Labotect C200
Centrifuges	Beckman GS-15R
	Inflexible rotors: GSA, GS3, SS34
	Swinging rotor: HB4
	Sorvall Superspeed RC5C
	Heraeus Biofuge fresco
	Sigma 3K20
	Heraeus Megafuge 1.0
Confocal microscopy	Beckman GS-15R
	Heraeus Megafuge 1.0
Cooling Centrifuges	Sigma 3K20

Dionized Water Supply	Heraeus Biofuge fresco
Fast prep MP	MP Biomedcals
Flow Cytometer	Sorvall Superspeed RC5C
Homogenizer	Inflexible rotors: GSA, GS3, SS34
In vivo luciferase imaging	Xenogen IVIS system, Caliper
Microwave	Whirlpool
Microscopes	Zeiss, Jena, Germany
	Leica Labovert FS, Nikon TMS
Micropipettes	Gilson
PCR-Thermocycler Veriti 384 well	Applied Biosystems, Foster City, USA
PCR-Thermocycler PTC-200	MJ-Research/Biometra, Oldendorf, Germany
PCR Machine	T3 Thermocycler, Biometra
pH meter	Beckman
Pipetboy	Pipetboy IBS Integra Biosciences
Power supplies	Gibco BRL ST 504 Biorad Power PAC 200 Biotec-Fischer Phero-STAB 550
Shaker	Heidolph
Sterile Work Benches	Steril Gard Class II Type A/B3, Baker Company Hersafe, Heraus
Thermomixer	Eppendorf
Thermocycler	T3 Thermocycler, Biometra
U.V Chamber	Hanau
Vortex	Scientific Industries Vortex Genie 2

Weighing	Sartorius
4 °C refrigerator	Liebherr
-20° C freezer	Liebherr
- 80°C freezer	Thermo Forma

2.1.2 Plasmids and Oligonucleotides

Table 2. Plasmids

Plasmids	**Nr.**	**Features**
Luc3rTA2E	2375	This is an autoregulated tetracycline inducible expression cassette.
pCAGGSflpE	2703	This vector expresses Flp recombinase driven by CAG promoter. The CAGGS promoter drives expression of FlpE.
pCMV rtTAHTV3	3362	This vector encodes rtTA2 fused with HTV3.
pCMVRTA2HYG	2288	This vector expresses rtTA2 under CMV promoter and also a hygromycine resistance. The vector was produce by T.May, 2002, HZI.
PCR-Blunt	K2700-20	Invitrogen
pCRE PAC	1930	This vector expresses Cre expression plasmid where Cre transcription is driven by the MC1 promoter a synthetic HSV-tk promoter and enhancer (Taniguchi et al., 1998)
pFB-ZB		This vector encodes full length of TET1 and was kindly donated by Prof. Zhang Yi.
pTRETight	3891	Clontech

TetLuc	3980	This vector encodes Tet promoter driving luciferase.

Table 3. Oligonucleotides

Oligonucleotides	Nr.	Sequence	Purpose
Actfwd	P3188	tggaatcctgtggcatccatgaaac	PT-PCR
Actrev	P3189	taaaacgcagctcagtaacagtccg	PT-PCR
Bi-GFPFW	P5103	cgagtaggcgtgtacggtg	PT-PCR
Bi-GFPRE	P5104	gctcctcgcccttgctcac	PT-PCR
Bi-LUCFW	P5105	gtttttggcgtcttccatgg	PT-PCR
Bi-LUCRE	P5106	cgtcagatcgcctggagacgc	PT-PCR
cLuc fw	P5108	gtttttggcgtcttccatgg	PT-PCR
cLuc re	P5109	cgtcagatcgcctggagacgc	PT-PCR
creI	P3018	gcctgcattaccggtcgatgcaacga	Genotyping
creII	P3019	gtggcagatggcgcggcaacaccatt	Genotyping
EMCV2	P3432	gccacgttgtgagttggata	Genotyping
EMCVNeo1	P5110	aagagtcaaatggctctcctcaagcgtatt	Genotyping
EMCVNeo2	P5111	gtctgttgtgcccagtcatagccgaatag	Genotyping
FUSIOFW	P5112	ctgagacaagcaattgagctg	Cloning
FUSIORE	P5113	tgagagctcttcccttcctt	Cloning
GFP1	P5114	agctgcccgtgccctggccc	PT-PCR
GFP2	P5114	tgtactccagcttgtgcccc	PT-PCR
INSU1FW	P5116	gctgagttggctgctgccac	Cloning
INSU1RE	P5117	ccgtatcccccaggtgtctg	Cloning
INSU1seFW	P5118	gtgggaggcctatataagcagagc	Cloning

INSU1seRE	P5119	gggagggacgtaattacatccctg	Cloning
INSU2chFW	P5120	ggcactctgtcgataccccac	Cloning
INSU2chRE	P5121	gaggaaagcgatcccgtgcc	Cloning
INSU2seRE	P5122	gagtaaactcgggctatggcagg	Cloning
LucRTfwd	P5071	gctgggcgttaatcagagag	PT-PCR
LucRTrev	P5070	gtgttcgtcttcgtcccagt	PT-PCR
neorev2	P1523	gtcatagccgaatagcctctcc	Genotyping
PGK2fwd	P4219	ctagtctcgtgcagatggac	Genotyping
Rttaful 1	P5123	tacccggggagcatgtc	Cloning
Rttaful2	P5124	atgtctagactggacaagagca	Cloning
TETALLfw	P5125	aaaatctcgagggatctggatctggatctgaag gaacaggaagctgca	Cloning
TETALLre	P5126	aaaatgcggccgcctatggtgtgataaattggc	Cloning
TETsepse RE	P5127	tggaactaatcatatgtggcct	sequencing

2.1.3 Enzymes, chemicals medium and Kits

Table4. Enzymes, chemicals medium and Kits

Enzymes, chemicals medium and Kits	**Supplier**	**Catalogue nr.**
50mM ß-Mercaptoethanol (500x)	GIBCO	31350
5X RLB buffer	Promomega	E1500
Albumin antibody	Abcam	ab106582
Alkaline Phosphatase, Calf Intestinal (CIP)	NEB	M0290L
Ascorbic acid	Sigma Aldrich	A4403
Azacytidine	Sigma Aldrich	A2385-100MG
Decitabine	Sigma Aldrich	A3656-10MG

DNA Loading Buffer Blue(5x)	Bioline	BIO-37045
DNA/RNA AllPrep Kit	Qiagen	80204
Doxycycline	AppliChem GmbH	A29951,0025
Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (High Glucose)	GIBCO	61965
FCS	Sigma	F7524
Gelatin solution 2%	Sigma	G1393
Genomic DNA isolation	Qiagen	51306
Glumax	Gibco	35050-038
HMM Maintenance Medium	Lonza	CC-3197
Inhibitor of glycogen synthase kinase 3 ß	Stemgent	04-00004
Insulin	Sigma	10516
IsoFlo®	Allbrecht GmbH	701-005-301
Klenow Fragment (3'→5' exo-)	NEB	M0212M
Knockout™ DMEM	Gibco	10829-018
KnockOut™ Serum Replacement	Gibco	10828-028
Lipofectamine 2000	Lifetechnologies	11668019
Luciferin	Synchem OHG	S039
MEM Non-Essential Amino Acids (100X), liquid	GIBCO	11140
Mitogen-activated protein kinase inhibitor(MAPKI)	Stemgent	04-00006
Monothioglycerol (MTG)	Sigma	M6145
Paraformaldehyde	Sigma	P6148-5KG
PCR Master Mix	p.j.k.	302004
Pen/Strep	Gibco	15140-122

Phusion® High-Fidelity DNA Polymerase	NEB	M0530S
Saponin	Sigma	47036
Sodium butyrate	Sigma Aldrich	B5887-250MG
Sodium Pyruvate MEM 100 mM,(100x) liquid	GIBCO	11360
SYBR® Green PCR Master Mix	Life Technologies	4309155
T4 DNA Ligase	NEB	M0202M
Valproic acid	Sigma Aldrich	P4543-25G
Vimentin Antibody	Sigma	V6630

2.1.4 Cell lines, mouse lines and bacterial strains

Murine cell lines

Murine G4B12 ES cells: G4B12 mES cells, Hybrid ES cell line from Bl/6 and 129/ola, targetable in the Rosa 26 locus (Haenebalcke et al., 2013).

NIH3T3: embryonic mouse fibroblast (MEF) cell line, ATCC CRL 1658.

MEF: primary murine embryonic fibroblasts isolated from the different mouse strains to serve as a feeder layer for murine ES cell culture.

Human cell line

HEK293T: human embryonic kidney cell line transformed by adenovirus type 5;, expressing SV40 large T antigen constitutively.

Bacteria strain

TOP10 E. coli: TOP10 F- mrcA Δ (mrr-hsdRMS-mcrBC) Φ80dlacZM15, ΔlacX74 recA1

araD139 Δ (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG

2.2 METHODS

2.2.1 *In vitro* methods

2.2.1.1 Agarose gel electrophoresis

1g of agarose were poured into microwavable flask along with 100mL of 1x TAE (40 mM Tris/acetate pH 7.5,20 mM Sodium Acetate, 1 mM Ethylenediaminetetraacetic acid). Then, the mixture was microwaved for 1-3min until the agarose was completely dissolved. Afterwards, the agarose solution was put stilly to cool down for 5min. Ethidium bromide (10 mg/ml) was then added to an appropriate volume agarose to a final concentration of approximately 0.2-0.5 μg/mL and poured into a gel tray with the well comb. The gel was placed into an electrophoresis chamber filled with 1x TAE when it got solidified. DNA samples were mixed with 5x loading buffer (Bioline) and loaded onto the gel. Meanwhile a molecular weight ladder (Hyperladder I, Bioline) was loaded to determine the size of DNA samples. The gel was running at 110V until the dye line was approximately 80% of the way down the gel. Gels were analyzed at last under UV-light (360nm).

2.2.1.2 Restriction analysis

Procedures for digesting DNA were followed by the manufacturer (NEB) and varied depend on the enzymes.

Total Reaction Volume	50 μl
DNA	1 μg
10x NEBuffer	5 μl (1x)
Restriction enzyme	1 μl
Incubation time	1 hour
H_2O	to 50 μl
Incubation temperature	Enzyme dependent

2.2.1.3 Cloning

TET1c-rtTA cloning

Backbone pCMV rtTAHTV3 (expressing transactivator rtTA, Nr. 3362) was digested with XhoI and NotI and 3536bp fragment was purified. Amplified TET1c from pFB-ZB encoding full length of TET1 (from prof. Zhang (Ito et al., 2010)) by introducing Xho1 and Not1 cutting sites. PCR products were purified and digested with XhoI and NotI and labled as TET1c. 3536bp fragment from the first step was then ligated with TET1c to the final vector TET1c-rtTA. To achieve a high fidelity of amplification, Phusion® High-Fidelity DNA Polymerase (NEB) was used for the PCR. The modified reaction and PCR protocol was followed. Primers for introducing XhoI and NotI cutting sites to TET1c fragment were TETALLfw and TETALLre.

Reaction protocol

Component	**50 µl Reaction**
Nuclease-free water	30 µl
5X Phusion HF	10 µl
10 mM dNTPs	1 µl
10 µM TETALLfw	2.5 µl
10 µM TETALLre	2.5 µl
Template DNA	2 µl
DMSO	1.5 µl
Phusion DNA Polymerase	0.5 µl

PCR protocol

STEP	**TEMP**	**TIME**
Initial Denaturation	98°C	30 seconds
35 Cycles	98°C 58°C 72°C	5-10 seconds 10-30 seconds 60 seconds

Final Extension	72°C	10 minutes
Hold	4°C	---

TET1c-U cloning

Backbone TET1c-rtTA was digested with NheI and SmaI. 5500 bp fragment was purified by gel and treated with Klenow enzyme (NEB) to blunt the hanging tail (rtTA binding site has been cutted away in this way). The blunted backbone was re-ligated again T4 DNA Ligase (NEB) and named as TET1c-unspecific.

R26-Tet-in-HCVsWT cloning

Two copies of the 1200bp chicken HS4 chromosomal insulator was cloned before and behind the Tet promoter, respectively in the PCR-Blunt vector by blunt ligation, named as PCR-BLUNT-INSULATOR. PTAREF1aTRE Tight 5TRSV vector which contains Tet promoter and HCV replicon was digested with Asis1 and MIu1and 15900bp fragment was kept as F1 for the next step. PCR-BLUNT-INSULATOR vector was also digested with AsisI and MluI and 2500bp fragment (insulator) was isolated and ligate with F1. R26-Tet-in-HCVsWT has been cloned. Then PCR-BLUNT-INSULATOR vector was cut by SwaI and AloI and the 5200 bp fragment was kept. The R26-Tet-in-HCVsWT was also cut by SwaI and AloI and the 18000bp fragment was kept. 5200 bp fragment and 18000bp fragment were ligated to the final TetinHCVasrWT vector. Sequences of the insertion fragment were characterized by DNA sequencing performed by the sequencing facility in HZI.

2.2.1.4 Dephosphorylation of 5´-ends of DNA using CIP in restriction enzyme reaction

Total reaction volume	20 µl
DNA	10 µg
10X NEBuffer	2 µl (1X)
CIP	1 µl

H_2O	3 µl
Incubation time	1 hour
Incubation temperature	37°

2.2.1.5 A-Tailing with klenow fragment (3'-->5' exo-)

Total reaction volume	50 µl
Purified blunt DNA	30 µg
10X NEBuffer 2	5 µl
Klenow fragment (3´→ 5´ exo–)	3 µl
dATP (10 mM)	1 µl
H_2O	11 µl
Incubation time	30 min
Incubation temperature	37°

2.2.1.6 Ligation of DNA fragments

COMPONENT	**20 µl REACTION**
10X T4 DNA Ligase Buffer	2 µl
Vector DNA	50 ng (0.020 pmol)
Insert DNA	500 ng (0.060 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

2.2.1.7 RT-PCR from tissues

Tissues preparation

RNAse free Eppendorf tubes and RNAse free tips and pipettes were used during the whole procedure.

Freezing organs

Mice were sacrificed by CO_2 and the organs were taken out and lanced into small pieces (25mg/tube), then transferred to prepared labeled freezing tubes as fast as possible due to RNA degradation. Afterwards, the tubes were immediately immersed into the liquid nitrogen and could be stored up to 1 year in -80°C.

RNA isolation

The frozen samples were taken out and placed on ice. The samples were transferred into homogenizing tubes filled with 2 homogenizing beads and 600µl RLT buffer (RNeasy Lysis Buffer, Qiagen) including ß-mercaptoethanol (10 µl β- mercaptoethanol/ ml RLT). Samples were then homogenized with the fast prep MP (MP Biomedcals, Cat.no.116004500) using the following settings: Time: 10sec, Rpm: 450. Homogenization was repeated for 3 times and in between steps samples were kept for 5 min on ice. The following procedures were followed according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen). Briefly, after 1 min 10000 rpm centrifugation under the fume cabinet, the samples were transfer to labeled 1,5 ml Eppendorf. 600 µl 70% ice-cold ethanol was added to the homogenizing products and the whole volume were then transfer to RNeasy spin column, which was placed in a 2 ml tube. Then the samples were centrifuged 1 min at 10000 rpm and flow through was discarded. 700 µl RW1(Wash Buffer, Qiagen) was then added to the samples and centrifuged 1 min at 10000 rpm. 2 times washing by 350 µl and 500 µl RPE respectively was preceded before the columns were placed in new tubes and centrifuge 1 min full speed again. At the end, RNA was dissolved in 35 µl RNAse free water. Concentration was determined by NANO drop.

cDNA synthesis

In 33µl reaction, 5 µg RNA was used for the synthesis. First diluted RNA was inoculated at 65° for 10 min followed by 2 min on ice. Then the reaction was initiated with the following protocol:

COMPONENT	**33 µl REACTION**

Beads tube	-
RNA	5 ng (in 25 µl)
Rnase free H_2O	25 µl
Oligo dT	3 µl

The mixture was incubated for and stored at -20°C.

RT-PCR

The primers for amplication Actin gene were put on the same plate as a control. The primer was used at the concentration of 10pmol. Pipetting scheme was prepared beforehand. Water controls were always included as the negative control.

COMPONENT	**20 µl REACTION**
Sybr green	10 µl
Primer forward	1 µl (10 pmol)
Primer forward	1 µl (10 pmol)
cDNA (1:5 dilution)	8 µl

2.2.1.8 Mammalian cell culture

Site-specific targeting by RMCE in cells

$5x10^5$ G4B12 mES cells were seeded on feeders on a gelatinized 6 well plate. Transfection was performed in the afternoon of the next day with Lipofectamine 2000 according to the manufacturer's instruction, briefly as following: 2,5 µg of pCflpe (# 2703, alternatively flpepuro: # 1825) and 2,5 µg of targeting vector were mixed with 250 µl pure DMEM. Then 10 µl Lipofectamine 2000 (Life Technologies) was added to 250 µl DMEM and inoculated for 5 min R.T. Both samples were then and incubated for 20 min at room R.T.

At last 500 µl mixtures were added to each 6 well. The transfected cells were inoculated with the mixture overnight. One day after transfection, the cells were transferred to a 10 cm^2 dish coated

with feeder cells. Two days after transfection, G418 or Puromycin selection was performed to the cells (0, 4 mg/ml for G418 and 300 U/ml for Puromycin).

Clones were visible at around 10 days post antibiotic selection pressure exposure.

ES cell lines and mouse strains

Several ES cell lines and mouse strains have been established in our lab for Tet induced transgene expression. The cell lines used in this thesis are displayed in Table 5 (Thesis Kruse, 2013).

Locus	Construct	ES cell line	Mouse line	Transactivator
Rosa26	Bi-directional Tet promoter driving luciferase abd GFP	R26-BiTetGFP-ES	R26-BiTetGFP	rtTA under Rosa26 promoter
	Anti-sense uni-ditrctional Tet promoter driving Luciferase-HCV	R26-TetHCVasrWT-ES	R26-TetHCVasrWT x Lap-tTA	rTA from Lap-tTA mouse
	Sense uni-ditrctional Tet promoter driving Luciferase-HCV	R26-TetHCVsWT -ES	none	rtTA from EF1a promoter
	Sense uni-ditrctional Tet promoter driving Luciferase-HCV, flanking with cHS4 insulators	R26-Tet-in-HCVsWT-ES	none	rtTA from transient transfection
COL1A1	Sense uni-ditrctional Tet promoter driving GFP	COL-TetGFP-ES	COL-TetGFP	rtTA under ROSA26 promoter
	Sense uni-ditrctional Tet promoter driving Luciferase	COL-Tetlucsr-ES	none	rtTA under ROSA26 promoter
	Sense uni-ditrctional Tet promoter driving Luciferase-HCV	COL-TetHCVsrWT-ES	none	rtTA under ROSA26 promoter
Tigre	Sense uni-ditrctional Tet promoter driving Luciferase	Tigre-Tetlucs-ES	none	rtTA under EF1a promoter
	Sense uni-ditrctional Tet promoter driving Luciferase-HCV	Tigre-TetHCVsWT-ES	none	rtTA under EF1a promoter
8.10	Bi-directional Tet promoter driving luciferase abd rtTA	LUC 8.10	LUC 8.10	rtTA under bi-directional Tet promoter

Table 5. Transgenic ES cell lines and mouse cells relevant for the thesis. tTA: Tetracycline transactivator protein; rtTA: reversed Tetracycline transactivator protein. Uni-directional Tet promoter indicates the synthetic promoter consists of 7 tandem operators and a minimal CMV promoter. Bi-directional Tet promoter represents the synthetic promoter compromises 1 CMV mini promoter fused to double 7 tandem operators on each flank, which could drive 2 irrelative genes at the same time in the presence of Doxycycline. GFP is Green fluorescent protein which is commonly utilized as reporter gene. HCV replicon denotes the Hepatitis C virus genome without Envelope proteins-

coding part. LUC8.10 mice carry a single integration of Luc3rTA2E (#2375) linearized with PvuI in IB10 (sub-clone of E14; strain: 129/OlaHsd) ES cells in the Luc8.1 locus.

Cell culture conditions

HEK293T cells, murine R26-Tet-in-HCVasrWT fibroblasts and murine NIH3T3 cells were cultured in DMEM (Gibco) medium supplied with 45 ml Fetal Bovine Serum (Sigma), 4.5 ml MEM Non-Essential Amino Acids (Gibco), 4.5 ml Penicillin Streptomycin (Gibco), 4.5 ml Glutamine (Gibco), 900 µl 2-Mercaptoethanol (Gibco). These cells were in cultivated at 37°C with 5% CO2 and 21% O2 at maximal humidity and fed every 3–4 days. Confluent cells were passaged into new culture plates or flasks containing fresh media at the ratio of 1:10 to 1:20 depending on the cell types. For passaging, cells were first washed with PBS and then trypsinized for 5 minutes. Afterwards, the trypsinization was inactivated by adding diluted FCS containing medium. Then the cells were transferred to a new culture flask or plate containing fresh medium. To determine the cell number, an aliquot of the cell suspension was counted.

Murine G4B12 ES cells, R26-BiTetGFP-ES murine embryonic stems cells, Tigre-TetHCVsrWT-ES cells and Tigre-Tetlucs-ES cells cell lines were cultured on irradiated mouse embryonic fibroblast (MEF) feeder layers in gelatin-coated plates. ES cell culture medium used in this thesis was Knockout medium: 400 ml knockout-DMEM, (Invitrogen) supplemented with 75 ml knockout serum replacement (Invitrogen), supplemented with 10 ml glutamine (Invitrogen), 5 ml nonessential amino acids (Invitrogen), 0.1 mM ß-mercaptoethanol (Sigma), 5 ml sodium pyruvate MEM 100 mM (100x, Gibco), 5 ml Penicillin Streptomycin (Gibico), 5 µg/ml insulin, 1000 U/ml and leukemia inhibitory factor (LIF), 1µl Mitogen-activated protein kinase inhibitor (Stemgent) and 3 µl inhibitor of glycogen synthase kinase 3ß (Stemgent). ES cells were cultivated at 37°C in a humidified atmosphere with 2% O2 and 7% CO2 and were fed daily. In respect to the rapid growing speed these mES cells were passaged every another day to keep them at optimal densities to avoid differentiation. During passaging it is critical to trypsinize the cells well to ensure a single cell suspension. The passage ratio was kept between 1:6 to 1:10 depending on the original density and growing speed. Overgrowing will sharply decrease the quality of ES cells so it is important to control cells in the middle size to avoid differentiate.

Hepatocytes derived from *in vitro* differentiation from Tigre-TetHCVsrWT-ES cells and Tigre-Tetlucs-ES cells were cultured in HMM Maintenance Medium (Lonza) in collagen coated plates at 37°C in a humidified atmosphere with 5% CO2 and 21% O2.

Endoderm cells derived from in vitro differentiation from Tigre-TetHCVsrWT--ES cells and TigreTetlucs ES cells were cultured in IMDM supplied with 20% FBS (Gibco), 1% Pen/Strep L-Gln,1% Non-essential aminoacids, 0,2% 50 mM ß-mercaptoethanol and 0,1% 450 mM Monothioglycerol (MTG)(Sigma) in collagen coated plates at 37°C in a humidified atmosphere with 5% CO2 and 21% O2.

2.2.1.9 In vitro differentiation

In vitro differentiation towards hepatocytes:

50 x 20 µl drops (each containing 800 ES cells) in mouse ES-cell medium without LIF were placed onto the inner side of the lid of a 10cm dish, and the plate was filled with 10 ml of sterile PBS. These hanging drops were cultivated for 3-5 days until embryoid bodies (EB) appeared. 25 EBs were collected and placed onto one gelatinized well of 6-well plate in 2 ml of IMDM. These EBs were cultured for 7-10 days and medium were exchanged every another day. Beating cardiomyocytes should come up spontaneously after 7 days. The cells were trypsinized with 500ul trypsin and stopped with 2ml IMDM per well, then transferred to a collagen-coated 6 well plate. Medium was changed to HMM next day. The cells were then cultivated for proximally 20 days in HCM and medium were exchanged every another day until the hepatic like cells appeared.

In vitro differentiation towards fibroblasts:

50 x 20 µl drops (each containing 800 ES cells) in mouse ES-cell medium without LIF were placed onto the inner side of the lid of a 10cm dish, and the plate was filled with 10 ml of sterile PBS. These hanging drops were cultivated for 3-5 days until embryoid bodies (EB) were appeared. 25 EBs were collected and place them onto one gelatinized well of 6-well in 2 ml of DEME. These EBs were then cultivated for proximally 15 days in DEME medium and were fed every another day until the fibroblast like cells appeared.

2.2.1.10 Immunostaining

Cells were cultured on cover slips in 12-well plates to 90% confluence. The medium was aspirated and the plates were washed 3 times with 2 ml PBS. 1ml/well 4% Paraformaldehyde (Sigma) was added for 10 min at R.T. for fixation. Afterwards the plates were rinsed 3 times briefly with PBS and the cells were then permeabilized with 0.1% Triton X-100 for 10 min at R.T. The plates were then washed 3 times with PBS and permeabilizion was block with 3%BSA at 4°C overnight. The next day, primary antibody were diluted in 3% BSA/0.1% Saponin (Sigma) solution to appropriate dilution and incubate for 60 min at R.T. (Albumin antibody from Abcam (1:1000), Vimentin Antibody from Sigma-Aldrich(1:800)). Then 3% BSA/0.1% Saponin solution was added to wash the plates for 3 times. The secondary Ab (1:1000) was then added for 1h at R.T. At last 3% BSA/0.1% Saponin solution was added to wash th plates for 3 times and the coverslips were at last mounted with mounting medium and invert onto glass slides. The mounted glass slides were left overnight at R.T. and analyzed the next day.

2.2.1.11 In vitro luciferase expression assay

1 x 10^5 cells were plated into 6 well plate per well and adhered until 90%-100% density was reached. The plates were washed 2 times with 2 ml PBS/well and 500ul 1x RLB buffer (Reporter Lysis Buffer, Promega, Cat.# E3971) were added for lysis. The plates were frozen in -80°C for 10 minutes and thawed on ice. Meanwhile, the illuminometer tubes were filled with 100 µl of the Luciferase Assay Reagent. 20 µl supernatant of the cells was applied to the illuminometer tubes which were transferred to the illuminometer later. Quantified luminance was related to the total amount of protein. Bradford assay (a spectroscopic analytical procedure used to measure the concentration of protein in a solution) was used for the analysis (Bradford, 1976).

2.2.1.12 Flow cytometry

1 x 10^7 cells were resuspended in 500 µl ice-cold FACS buffer (10%FCS in PBS) and re-suspended thoroughly to guarantee a single-cell suspension form of the cells. Flow cytometry (BD FACScalibur, FACSaria, FACScanto) was used to analyze GFP expression (level and

population) in transgenic ES cells and sort for TET1c-rtTA positive cells for the further analysis analysis. FlowJo 7.6 software was used for the data analysis.

2.2.1.13 In Vitro chemical treatments

Murine ES cells were seeded 1 x 10^5 cells in a 6-well plate. Doxcycline was dissolved in ethanol to stocking concentration of 2.5 mg/ml. And the working concentration was 1:1000. Treatment was performed at least 24 hours before measurement. Dnmt inhibitors azacytidine (Sigma Aldrich) was dissolved in ethanol and added at a final molecular concentration of 1 μM for 72 hours. Ascorbic acid (Sigma Aldrich) was dissolved in PBS at the concentration of 1 mg/ml. 1:10 dilution was used in the treatment. Treating time varied accordingly to experiments plan. Decitabine (Sigma Aldrich) was dissolved in ethanol and added to the medium at the final molecular concentration of 0.5 μM and the treatment was lasted for 48 hours.

2.2.2 *In Vivo* methods

2.2.2.1 Mice breeding and handling

ROSAGFP mice were generated from murine embryonal stems cells upon targeting the pEM-rTA2luc3eGFP vector into the Rosa26 locus by RMCE (Sandhu et al. 2013). Luc 8.10 mice were generated by random introduction of a single copy of the vector pEM-rTAluc (Pamela Riemer, Thesis). The mice were bred in house at the facility of HZI in SPF (specific-pathogen-free) conditions.

All mouse procedures were conducted under local ethical guidelines and after obtaining the permissions.

2.2.2.2 Hydrodynamic tail vein injection

5-12 weeks old ROSAGFP and Luc 8.10 mice were chosen for the experiments. Before injection body weight of each mouse has been measured and the tails of the mice were warmed by a heating lamp (120W bulb) for 2-5 minutes. When the vein dilated and became more visible the

mice were directly placed into the restraint chamber for fixing during the injection. The area was swabbed with alcohol and the needle was inserted into the tail vein. 25µg plasmid DNA diluted in PBS (1/10 bodyweight ml) were injected for each mouse in 2-5 seconds.

2.2.2.3 In vivo differentiation of ES cells to teratoma

1 x 10^6 embryonic stem (ES) cells were cultivated on a gelatinized T75 flask without feeder. On the day of injection the cells were trypsinized and stopped with ES medium. The cells were then centrifuged down at 1000 rpm for 5 min and re-suspended to a cell number of $1*10^6$/100 µl-$1*10^7$/100 µl in ice-cold PBS (cells were kept on ice all the time). Afterwards 100 µl cells were injected subcutaneously with a 26 gauge needle on the lower flanks of 8-12-week-old mice on the both sides. Mice were sacrificed 5 weeks after injection of the cells to isolate the teratomas.

2.2.2.4 Bioluminescence imaging with Xenogen IVIS 200 (In vivo luciferase expression)

To quantify the luciferase expression level in transgenic mice, Xenogen IVIS 200 Imaging Series (Caliper) was used. This system allows using real-time, noninvasive imaging to monitor and record cellular activity of luciferase *in vivo*. Mice for the experiments were anesthetized in the built-in anesthetic chamber by 2-2.5% isoflurane (Albrecht). Afterwards each mouse was injected with 100 µl of luciferin (30 mg/ml in PBS, Synchem OHG) respectively by intraperitoneal (i.p.) injection. Then the mice were transferred to imaging chamber. Standard setting was used to detect the luciferase expressing level except the changing of the "field" from "C" to "D" and the scanning time prolonged to 5 min. Living image 2.60.1 (Igor Pro 4.09A) software was used to analyze images. The photons produced in the subject diffuse through the tissues and the IVIS determines this signal at the surface of the subject by means of a sensitive photon capturing CCD (charged-coupled device) camera.

2.2.2.5 In vivo chemical treatment

Doxycycline (Sigma): 2 mg/ml; stock: 50 mg/ml in water, kept at 4°C. Doxycycline was administered via the drinking water. The water was changed every 3 days.

Luciferin: 100 µl of 30 mg/ml Luciferin in PBS (Synchem OHG, stored at -70°C) were injected i.p.. Images were taken at consecutive time points to cover the time when distribution was optimal and the signal the strongest (between 3 and 10 minutes post injection).

2.2.2.6 Histological analysis

Mice were sacrificed and livers were fixed in formalin 4% for 24 hours. Micrograph preparation and GFP staining was performed by histopathology facility of HZI. The quantification of GFP was determined by counting the number of stained cells by microscopy.

3 RESULTS

3.1 Evaluation of Tet cassette expression in different loci

Doxycycline induced Tet cassette expression in mice is of high interest. Few integration sites have been reported for doxycycline controlled expression with variable success; however, since different reporters and/or cassette designs were used, the performance of the individual Tet cassettes could not be directly compared. Thus, the first aim of this thesis was to compare three particular integration sites for Tet cassette expression in ES cells. The loci were chosen based on i.) Previous reports showing doxycycline dependent expression and ii.) Their availability for targeted integration by means of Flp mediated recombinase mediated cassette exchange (RMCE). The chosen loci are the Rosa26 locus (available for RMCE in RosaAntiluc/G4B12 cells (Sandhu et al., 2011), the COL1A1 locus (available for RMCE in KH2 cells (Beard et al., 2006b) and the Tigre locus (Zeng et al., 2008) which was made available for RMCE in ES cells by transferring the Tigre locus to ES cells (chapter 1.2.2.1 and Wirth et al., umpublished). For the map of these loci as well as the details of the targeting procedures see supplementary Figure 1.

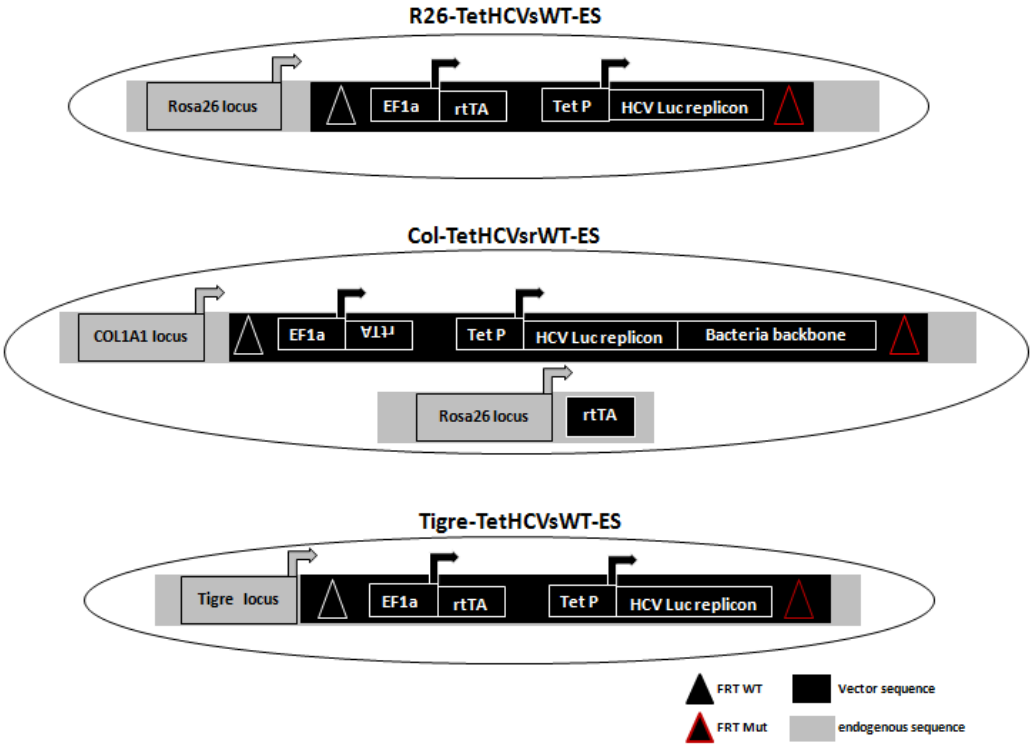
3.1.1 The Tigre locus supports best expression of Tet driven transgene expression in ES cells

To compare Tet cassette expression in these three loci of embryonic stem cells, a luciferase reporter cassette was targeted into the three genomic sites. This reporter cassette comprises the luciferase gene, which is expressed from a Tet promoter in a bicistronic unit together with the subgenomic HCV replicon (the HCV replicon is not relevant in this study) (Figure 9a-b). For targeting the Rosa26 locus and the Tigre locus, the plasmid (TetHCVsWT) encodes a cassette for constitutive expression of the rtTA reverse transactivator, which is required to specifically activate the Tet promoter in a doxycycline dependent manner. Since KH2 ES cells constitutively express rtTA under the control of the Rosa26 promoter, a modified cassette (TetHCVsrWT), that comprises an inactivated (reverted) rtTA coding sequence, was targeted in this particular site.

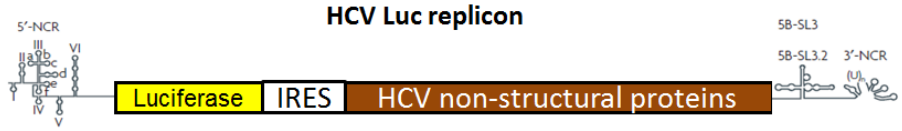
Targeting was achieved upon lipofectamine transfection of ES cells. Correctly targeted cells were identified by selection for G418 resistance and confirmed by PCR (data not shown). According to the site of integration, the ES cell clones were designated as R26-TetHCVsWT-ES, Col-TetHCVsrWT-ES and Tigre-TetHCVsWT-ES, respectively (Figure 9A). Individual clones of each integration site were propagated and analyzed for the transgene expression level by monitoring luciferase activity.

$1x10^5$ cells of each targeted ES cell clone were cultivated with or without doxycycline (2.5 µg/ml) for 48 hours. As a positive control, the expression level from the non-integrated (episomal) state of the cassette was evaluated. To this end, the wild type ES cells were transiently transfected with the TetHCVrWT construct in presence/absence of doxycycline. 48h later, the cells were harvested and analyzed for luciferase expression. The luciferase levels were normalized to total protein content of the cells.

a.)



b.)



c.)

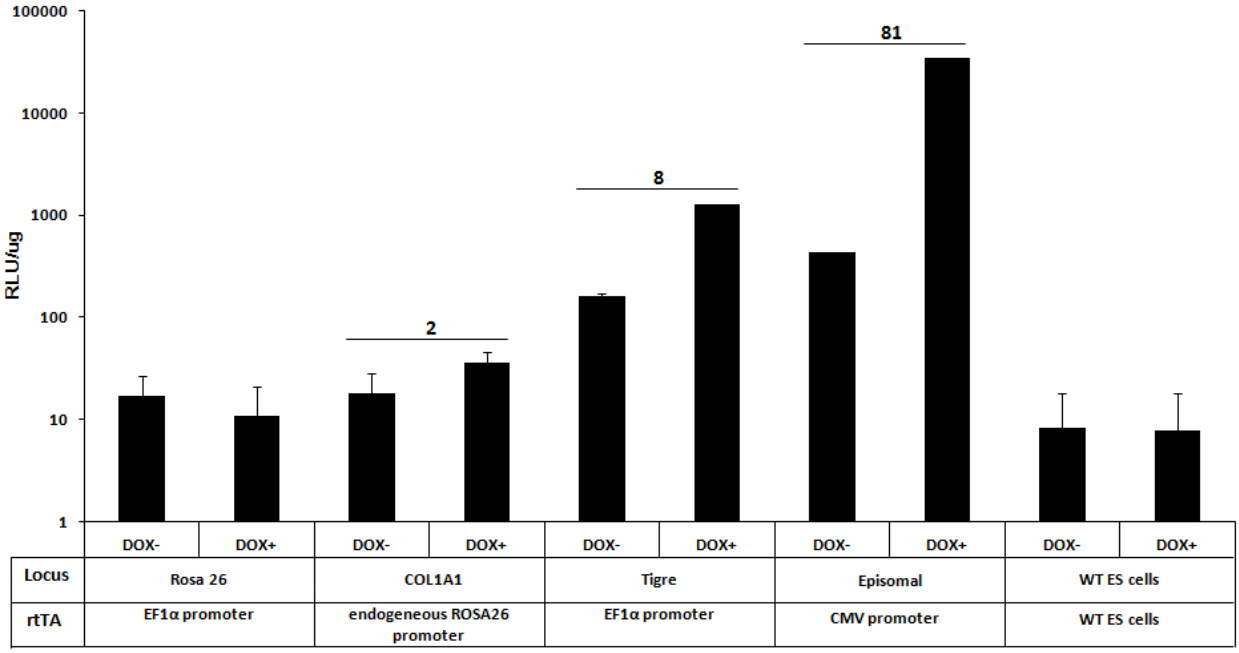


Figure 9: Evaluation of three chromosomal loci for the expression of Tet cassette. a.) Schematic depiction of the R26-TetHCVs-WT-ES, COL-TetHCVsrWT-ES and Tigre-TetHCVsWT-ES cell clones after targeting. All loci were targeted with the luciferase reporter cassette HCV Luc as specified in b). The transactivator in the R26-TetHCVsWT-ES cell line as well as in the Tigre-TetHCVsWT-ES cell line is encoded by the targeting cassette and driven by the EF1α promoter. In the COL-TetHCVsrWT-ES cell clones the transactivator is driven by the Rosa26 promoter as depicted. b.) Depiction of the luciferase encoding part of HCV genome and fused with luciferase as reporter constructs. This construct serves in this study as a luciferase reporter; the HCV part is not relevant for the study. c.) Luciferase expression was analyzed in populations of targeted cells as indicated. As control, wild type (WT) ES cells were used that do not encode a functional luciferase gene. Briefly, 1 x10^5 R26-TetHCVsWT-ES cells, COL-TetHCVsrWT-ES cells and Tigre-TetHCVsWT-ES cells were seeded on irradiated mouse embryonic fibroblast feeder cells in absence and presence of 2.5µg/ml doxycycline for 48h. To evaluate the episomal state, 1 x10^5 G4B12 ES cells were seeded on irradiated mouse embryonic fibroblast feeder cells. When the cells reached 90% confluence 1µg rtTA expression vector pCMVRTA2HYG and 1µg TetHCVrWT encoding Tet promoter driving HCV fused luciferase were co-transfected on these cells in the presence/absence of doxycycline. All the cells were harvested 48h after doxycycline addition. Then the cells were harvested and analyzed for luciferase expression. Luciferase activity observed in relative light units (RLU) was normalized to µg of total protein present in the cell lysate. Error bars indicate standard deviations calculated from 3 independent samples.

Figure 9c shows the evaluation of the luciferase reporter construct in the different loci. In presence of doxycycline, high luciferase expression of 1 x10^5 RLU/µg was observed for

TetHCVrWT episomal expression. In contrast, about 80-fold less expression was observed in absence of doxycycline which confirms that the construct was functional. However, neither basal expression nor doxycycline-mediated induction was observed when this Tet cassette was integrated into the Rosa26 locus. In the COL1A1 locus, in presence of doxycycline very low expression (8 $x10^1$ RLU/µg) was detectable with a fold induction of 2 compared to the non-treated cells. For the Tigre locus, basal levels were found to be elevated to 200 RLU/µg and 8-fold inducibility was reached upon cultivation in doxycycline containing media. Importantly, in presence of doxycycline 20- and 100-fold higher luciferase expression was detected for the Tigre locus comparing to the COL1A1 locus and the Rosa26 locus, respectively. However, in none of these loci the cassette could reach the expression level of the episomal state. As shown in Figure 9c, 10-fold more luciferase expression was observed in the episomal state compared to the expression level in the Tigre locus upon targeting. Together, this indicates that specific silencing of the Tet cassettes occurred after genomic integration into all three loci, although this occurred to different extent.

To rule out the possibility that the rtTA level driven by the EF1α promoter is not sufficient to induce the Tet promoter upon single copy integration by targeting, rtTA was additionally provided by transient transfection of the vector pCMVRTA2HYG. In this vector the CMV promoter controls the expression of rtTA. However, the overexpression of rtTA in R26-TetHCVs-WT-ES, COL-TetHCVsrWT-ES and Tigre-TetHCVsWT-ES cells did not result in increased expression levels of luciferase (data not shown). Thus, the expression level of rtTA is not the reason for the low expression of Tet cassettes after chromosomal integration.

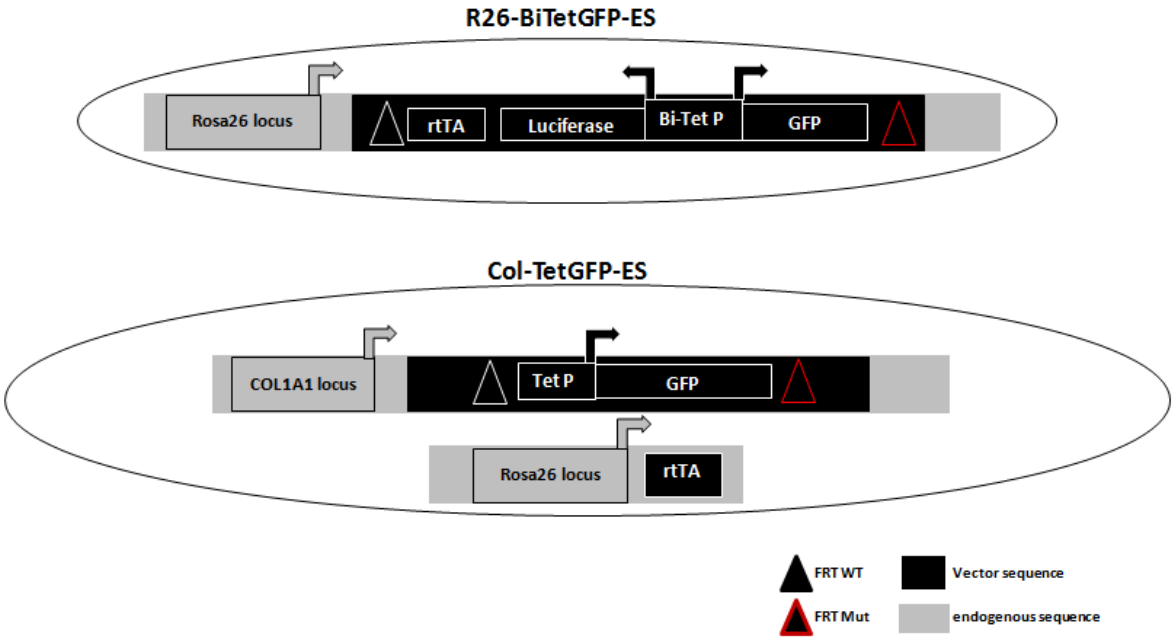
To sum it up, even though in the episomal state proper expression and regulation of luciferase was observed, the expression dramatically dropped upon integration of the Tet cassettes in all three investigated chromosomal sites. Of note, out of the three investigated loci, the Tet cassette performed best in the Tigre locus.

3.1.2 Single cell analysis shows heterogenous expression in the Rosa26 locus and in the COL1A1 locus

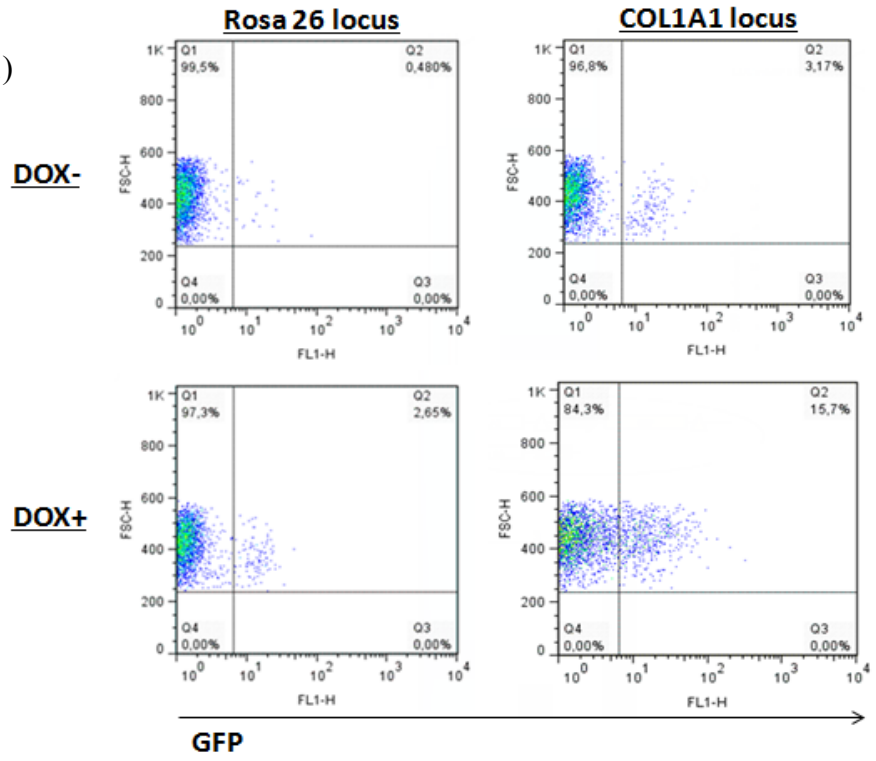
To further characterize the downregulation of expression upon chromosomal integration it was investigated whether the low expression was the result of low but homogenous expression levels in individual cells or the fact that a few cells in the population expressed high levels of luciferase while others were completely silenced. For this purpose a GFP reporter Tet cassette was integrated into the Rosa26 and the COL1A1 locus by RMCE. In contrast to luciferase, which is measured in the cell lysates and which allows to detect transgene expression in populations of cells, GFP allows a cell specific analysis of expression. The targeted ES cell clones were identified by PCR. The targeted transgenic ES cell lines (designated as R26-BiTetGFP ES and Col-TetGFP) were treated for 48h with or without doxycycline. GFP expression was analyzed by flow cytometry. In both cell lines, rtTA was expressed by the endogeneous Rosa26 promoter.

In Figure 10b, the expression profiles of representative cell populations are depicted. In absence of doxycycline, a small fraction of the two isogenic cell populations showed GFP expression (0.4% for the Rosa26 and and 3% for the COL1A1 locus). Interestingly, upon treatment with doxycycline, a fraction of cells from cell lines both showed GFP expression (2.6% for Rosa26 and 15.7 % for COL1A1 locus). Of note, the majority of cells do not express GFP after treatment with doxycycline. In Figure 10c, data are summarized for three populations per clone. In the Col-TetGFP ES cell line, a total of 15% of cells exhibited GFP expression after doxycycline induction. This corresponds to a moderate induction level (5-fold). In contrast, only 2.6% of the R26-BiTetGFP ES cells showed GFP signal after induction with doxycycline, which was 5-fold less compared to the Col-TetGFP ES cells. These data indicate that the cells were highly heterogeneous with respect to the expression and only a small fraction was in the active state after treatment with doxycycline.

a.)


R26-BiTetGFP-ES
Rosa26 locus
rtTA
Luciferase
Bi-Tet P
GFP
Col-TetGFP-ES
COL1A1 locus
Tet P
GFP
Rosa26 locus
rtTA
FRT WT
FRT Mut
Vector sequence
endogenous sequence

b.)


Rosa 26 locus
COL1A1 locus
DOX-
DOX+
Q1 99,5%
Q2 0,480%
Q4 0,00%
Q3 0,00%
Q1 96,8%
Q2 3,17%
Q4 0,00%
Q3 0,00%
Q1 97,3%
Q2 2,65%
Q4 0,00%
Q3 0,00%
Q1 84,3%
Q2 15,7%
Q4 0,00%
Q3 0,00%
FSC-H
FL1-H
GFP

c.)

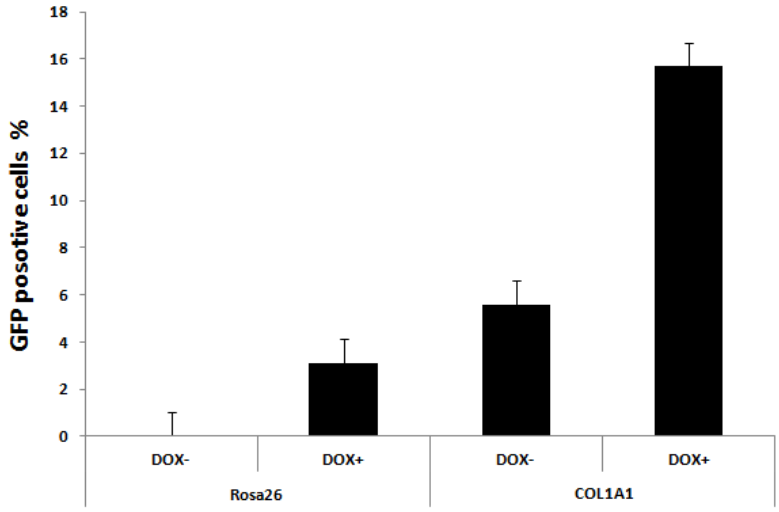


Figure 10: Heterogeneous GFP expression from the Tet promoter in mouse ES cells. a.) Depiction of the Tet cassettes integrated in R26-BiTetGFP ES cells or Col-TetGFP ES cells. The construct integrated into the R26-BiTetGFP ES cells carried both the luciferase and the GFP under a bi-directional Tet promoter, while the rtTA was expressed by the endogeneous Rosa 26 promoter. A similar construct was integrated into the Col-TetGFP ES cells carrying the uni-directional Tet promoter driving GFP in the COL1A1 locus and the transactivator rtTA was expressed by the endogenous Rosa26 promoter. b-c.) 1 x10^5 Col-TetGFP ES cells or R26-BiTetGFP ES cells were seeded on the irradiated mouse embryonic fibroblast feeder cells in absence and presence of 2.5 µg/ml doxycycline for 48h. Then the cells were harvested and analyzed for GFP expression by FACS. Data were analysed by FLOWJO (FLOWJO; LLC). Representative plots are depicted. c.) Data are shown for three populations per clone. Error bars indicate standard deviations.

To sum up the findings, COL1A1 locus and Tigre locus supported better Tet cassettes expression in ES cells compared to Rosa26 locus. However, the achieved expression levels were still low. Of note, single cell GFP expression analysis showed that the overall low expression of the Tet cassettes upon integration was the result of a heterogenous expression within the targeted population of cells. Only a small fraction of cells expressed GFP while the other cells seemed to be completely silenced.

3.1.3 Silencing of Tet cassette in COL1A1 locus and Tigre locus upon differentiation

To investigate whether the expression of the Tet cassettes could be maintained upon embryonic differentiation, transgenic mice were generated from Col-TetGFP ES cells and R26-BiTetGFP ES cells, respectively. They were analyzed for GFP expression by RT-PCR. For this purpose

animals from both strains were fed with doxycycline in the drinking water. The mice were sacrificed and the liver and the spleen were harvested for RNA extraction. However, no GFP expression was detectable in the samples of R26-BiTetGFP and Col-TetGFP transgenic mice (data not shown).

Since transgenic mice were not available for the Tigre ES cells, *in vitro* differentiation was followed to mimic embryonic development and to investigate the behavior of Tet cassettes in the Tigre locus. For this purpose, a differentiation protocol was used that induces differentiation upon withdrawal of the stem cell factor LIF and embryonic body formation by using hanging drops. Then, the cells, in embryonic bodies, were exposed to medium that supports the differentiation into the endodermal lineage and finally a hepatocyte-like state (for details of the protocol see chapter 2.2.1.9). Expression was analyzed at different stages of differentiation: in embryonic bodies and in the finally differentiated state.

To investigate the expression upon differentiation, ES cells were targeted with two different constructs. On the one hand the Tigre-TetHCVsWT ES cell clones were used. On the other hand a similar luciferase expression construct (Tetlucs), optimized for luciferase expression by elimination of HCV elements, was specifically targeted into the Tigre locus. These cells are designated as Tigre-TetlucS ES cells. For each cassette various clones were established.

In Figure 11b a scheme of the differentiation protocol and representative pictures of the cells at the various differentiation stages are shown. In particular, the changes of the morphology from ES cells (Figure 11b-A) via embryonic bodies (Figure 11b-B) and endodermal lineage (Figure 11b-C) to the hepatocyte-like cells (Figure 11b-D) can be observed. In this terminally differentiated stage, a typical hepatocyte-like phenotype was observed, characterized by distinct nuclei and a confluent monolayer (Figure 11b-D). The hepatic phenotype of the differentiated cells was confirmed by albumin staining by immunocytochemistry (Figure 11b-E).

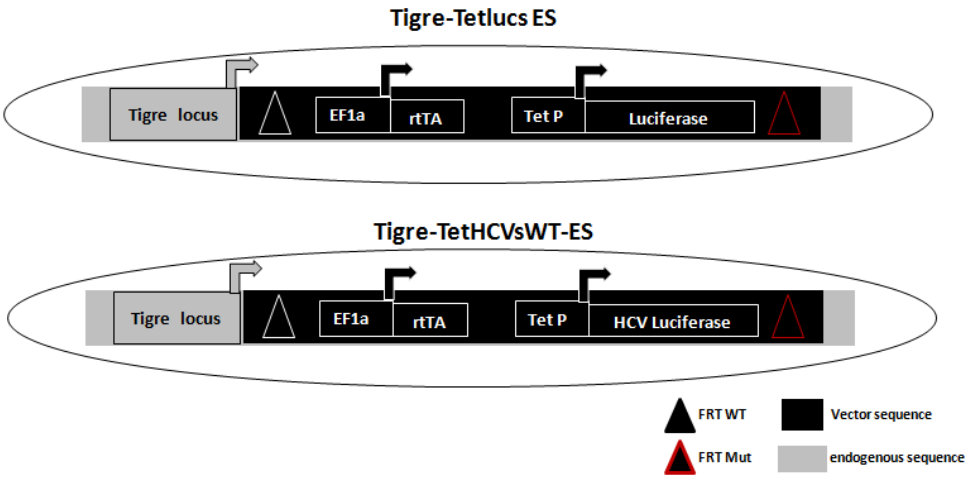
From all stages aliquots of cells were exposed to doxycycline for 48 hours and luciferase expression was analyzed.

All the ES clones showed an inducible expression for both of the cassettes. This is exemplified by the ES clone Tigre-TetlucS 2-5 and the Tigre-TetHCVsWT-ES clone 3-1 in Figure 11. As expected from the different design, the Tigre-TetlucS cells showed about 10-fold increased levels of expression upon doxycycline treatment. Interestingly, upon formation of embryonic

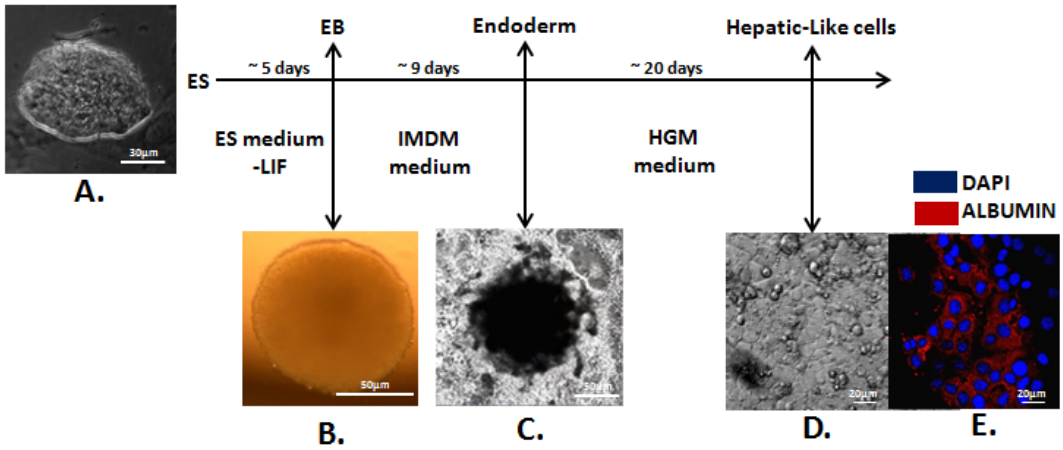
bodies and in terminally differentiated hepatocytes expression was found to be diminished or completely lost for both cassettes.

These data indicate that although the Tigre locus supports a good expression of Tet cassettes in ES cells, the expression of Tet cassette is lost after differentiation. This suggests that also in this integration site the Tet cassette is silenced after differentiation.

a.)



b.)



c.)

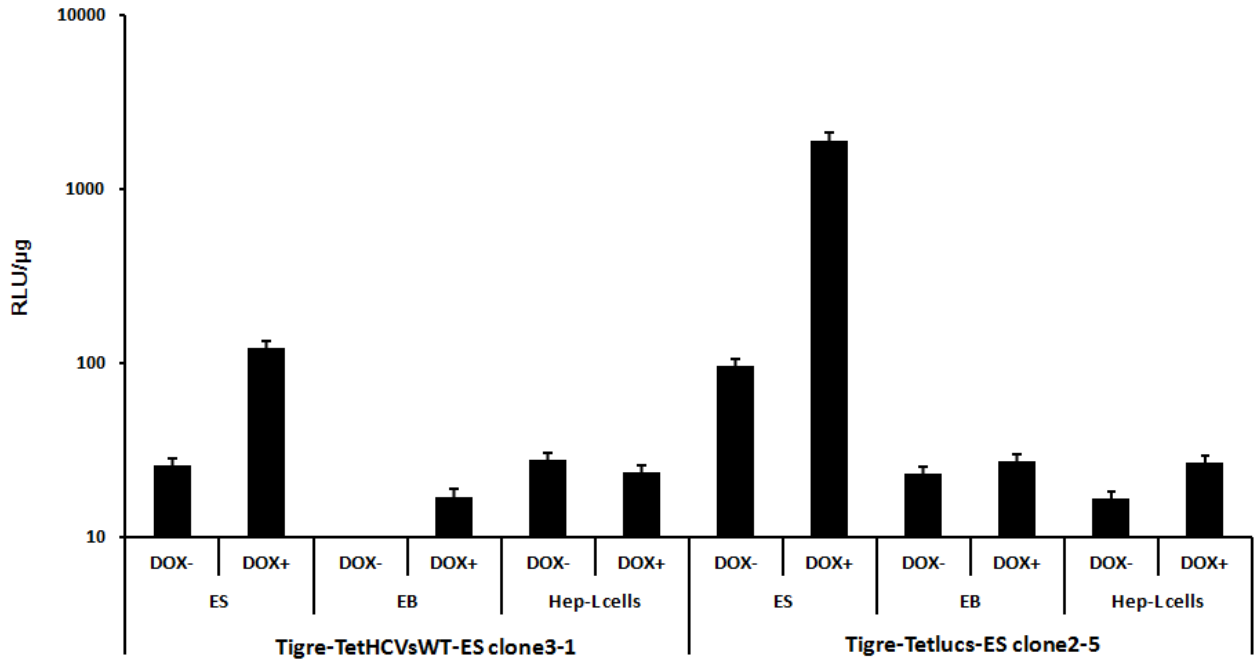


Figure 11: Tet controlled Luciferase expression in the Tigre locus upon *in vitro* differentiation. a.) The design of the cassettes integrated in the Tigre-Tetlucs-ES clone2-5 and Tigre-TetHCVsWT-ES clone3-1 is depicted. Tigre-Tetlucs ES cells contain luciferase. The expression is controlled by the Tet promoter. In contrast the Tigre-TetHCVsWT-ES cells comprise a HCV luc reporter driven by the Tet promoter. Both cassettes are integrated into the Tigre locus. b.) Procedures for *in vitro* differentiation: 50 x 20 µl drops (each containing 800 Tigre-TetlucS-ES cells or Tigre-TetHCVsWT-ES cells) in mouse ES cell medium without LIF were placed onto the inner side of the lid of a 10cm dish, and the plate was filled with 10 ml of sterile PBS. The hanging drops were cultivated for 3-5 days until embryoid bodies (EB) appeared. A representative picture is shown (B). 25 EBs were collected, dissociated and plated in 2 ml of IMDM on one gelatinized well of a 6-well plate to achieve the endoderm stage (C). Beating cardiomyocytes came up spontaneously after 7-10 days (not shown). Then, the cells were transferred to a collagen coated 6-well. Medium was shifted to HGM (hepatocytes growth medium) next day. The transformation of cell morphology was closely monitored every day. After proximally 20 days in HGM, hepatic like cells appeared (D). The phenotype of the hepatic like cells was confirmed by immunostaining of hepatic marker albumin (red), nuclei were stained with DAPI (blue) (Figure a-E). For comparison, a picture of ES cells is shown (A). c.) Luciferase activity was detected in relative light units (RLU) and normalized to µg of total protein present in the cell lysate. Error bars indicate standard deviation from 3 independent samples of a particular cell clone. For each construct, results from one out of five tested clones are depicted.

3.2 Transgene silencing can be rescued upon treatment with epigenetic modifiers

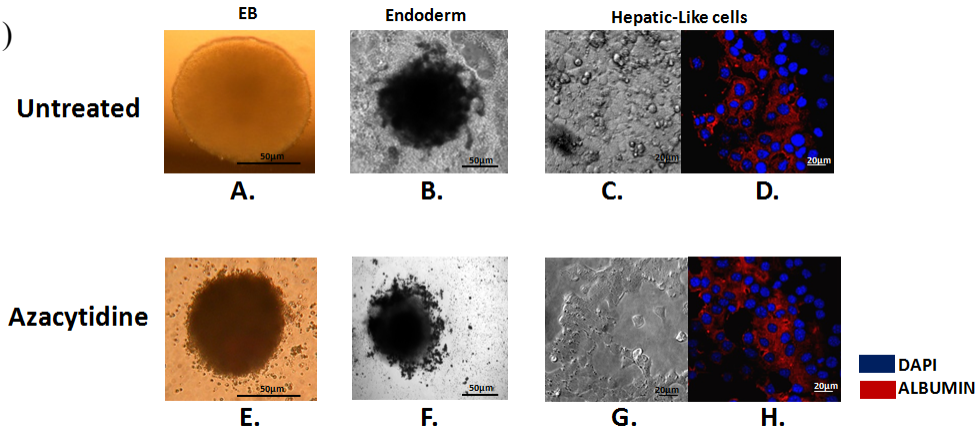
Previous data showed that the Tet cassettes were expressed to a moderate extent in the Tigre locus in ES cells but they were silenced upon *in vitro* differentiation. To investigate the mechanism of transgene silencing, epigenetic modifiers were used to rescue the expression. To this end, Aza was used to treat the cells during the whole period of differentiation. Aza is a demethylation reagent that specifically prevents the action of the DNA de novo methylase DNMT1 (chapter 1.2.1.2).

The two different ES cell clones, Tigre-TetHCVsWT-ES clone 3-1 and Tigre-TetlucS-ES clone 2-5, were analyzed. To monitor the effect of Aza on the differentiation, untreated ES cells were differentiated in parallel. Both conditions, with or without Aza treatment led to the formation of embryoid bodies as well as differentiation into the hepatocyte-like stage. However, Aza treated group showed an altered morphology characterized by a rough surface of embryoid bodies compared to the untreated group. Moreover, longer differentiation times and a reduced efficiency of hepatic differentiation were observed in the Aza treated group. Lineage specific differentiation into hepatocyte-like cells was confirmed by staining for albumin expression (Figure 12a-C, D and G, H).

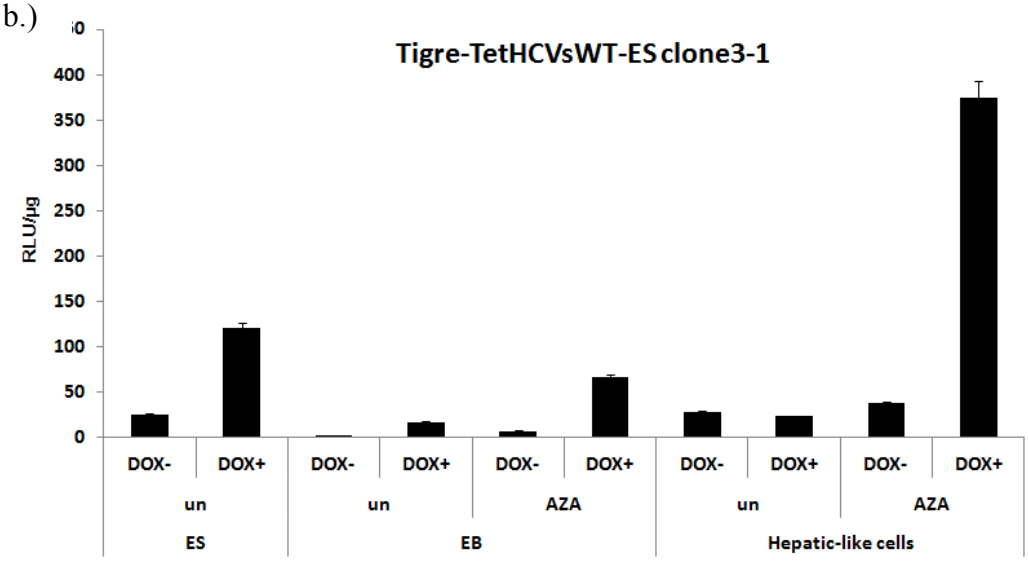
Interestingly, while the expression of luciferase was silenced in Aza-free control sampleswhich correlates with previous data (compare chapter 3.1.3), in presence of Aza both ES cell clones maintained the respective luciferase expression level during differentiation. This was verified for the embryoid body cells as well as in the hepatocyte-like cells. TheAza treated group of the Tigre-TetHCVsWT-ES clone 3-1, exhibited 6-fold and 20-fold more expression at EB stage and hepatic stage, respectively, compared to the untreated group. Similarly, Tigre-Tetlucs-ES clone 2-5 also showed a 30-fold and 8-fold higher expression level in the Aza treated group than in theuntreated group at EB and hepatic stage, respectively (Figure 12b). Overall, compared to the embryonic state the expression level could be partially maintaind during differentiation of the Tigre-Tetlucs-ES clone2-5 cells. Interestingly, the luciferase induction was even increased in the differentiated Tigre-TetHCVsWT-ES clone 3-1 at the hepatocyte-like differentiation stage (Figure 12 b).

In summary, the silencing of the Tet cassette during *in vitro* differentiation could be at least partially rescued by Aza treatment. This implies that the silencing of the Tet promoter upon integration into the Tigre locus might be the consequence of DNA methylation of the Tet promoter during differentiation and can be overcome by blocking its methylation.

a.)



b.)



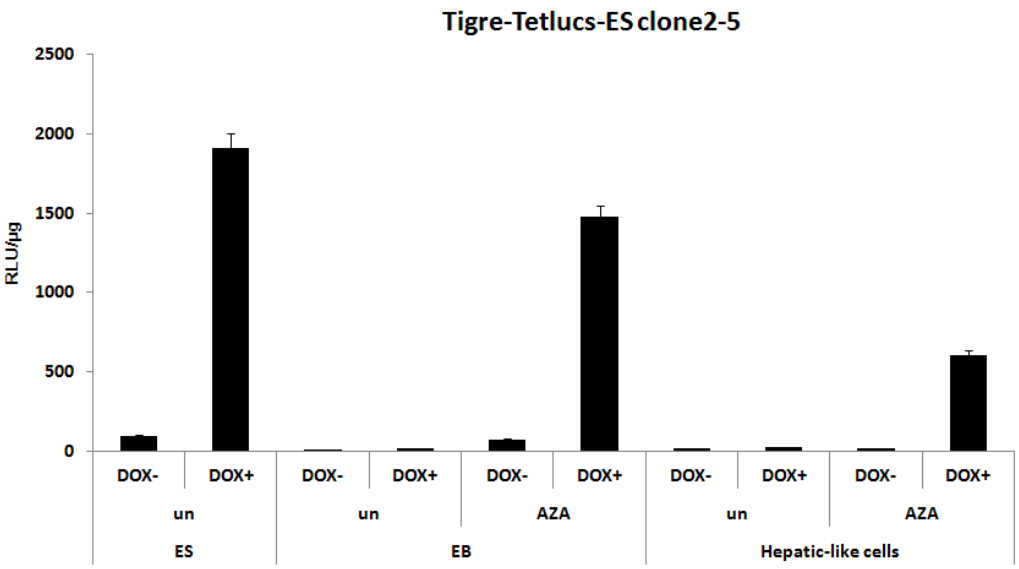


Figure 12: Expression level of Tet cassette in the Tigre locus upon *in vitro* differentiation to hepatic like cells in presence of 5-azacytidine. a.) Morpology of Aza untreated/treated samples during differentiation according to the protocol specified in chapter 2.2.1.9. b.) Luciferase expression levels during the differention. For the Aza treated group, Aza was present during the whole procedure. 2.5 µg/ml doxycycline was present/absent during the whole procedure. Luciferase activity observed in relative light units (RLU) was normalized to µg of total protein present in the cell lysate. Error bars indicate standard deviation from 3 independent samples.

3.3 Silencing of the Tet cassettes can be partially overcome by chromosomal insulators

3.3.1 Insulated construct expression in ES cells in Rosa26 locus

The results from the previous chapters suggest that the Tet promoter is epigenetically modulated upon integration into the three chromosomal sites. To shield the Tet promoter from the influences of the flanking chromatin, chromosal insulators were introduced into the targeting cassette. Chromosal insulators are described to prevent transgene silencing against the impacts from the genomic context by shielding the cassette. The best characterized insulator is the 1.2kb spanning cHS4 element, a vertebrate insulator element derived from the *chicken β-like globin* gene cluster (Majocchi, Aritonovska, & Mermod, 2014; Rincón-Arano, Furlan-Magaril, & Recillas-Targa, 2007; Sharma et al., 2012; Sinn, Burnight, Hickey, Gary, & Mccray, 2005).

Various combinations have been tested. For optimal shielding, it has been shown that two copies of cHS4 full length elements need to be introduced 5' and 3' of the cassette of interest (ekkali et al., 2008; Bell et al., 1999; Burgess-Beusse et al., 2002; Recillas-Targa et al., 2002). Accordingly, two copies of the full length cHS4 element were incorporated into the vector TetHCVrsWT to flank the Tet cassette (Figure 13a).

Since the expression of the Tet cassette was the weakest in the Rosa26 locus, this locus was chosen to test the modified Tet construct. Thus, the modified construct was targeted into the Rosa26 locus in ES cells by RMCE. Clones (designated as R26-Tet-in-HCVsWT-ES) were established and confirmed by PCR.

Upon analyzing 13 isogenic R26-Tet-in-HCVsWT-ES clones with respect to the expression level in presence/absence of doxycycline, four different expression profiles were observed.

Profile 1 as represented by R26-Tet-in-HCVsWT-ES CL5' exhibited a 60-80 -fold higher expression than the uninsulated ones (Figure 13 b). At the same time, a weak induction of only 2 was observed. This profile was found in 2 out of 13 clones.

4 out of 13 clones are classified to profile 2. This profile is displaying a better induction of luciferase expression upon doxycycline treatment than clones of profile 1. For instance, R26-Tet-in-HCVsWT-ES CL3' achieved 6-fold increase in luciferase expression upon doxycycline treatment however the expression level after induction is lower than that from profile 1.

Of note, 6 out of 13 clones were classified into profile 3. These clones showed neither elevated basal expression nor any induction compared to the non-insulator clones. This is exemplified by R26-Tet-in-HCVsWT-ES CL1' and CL4'.

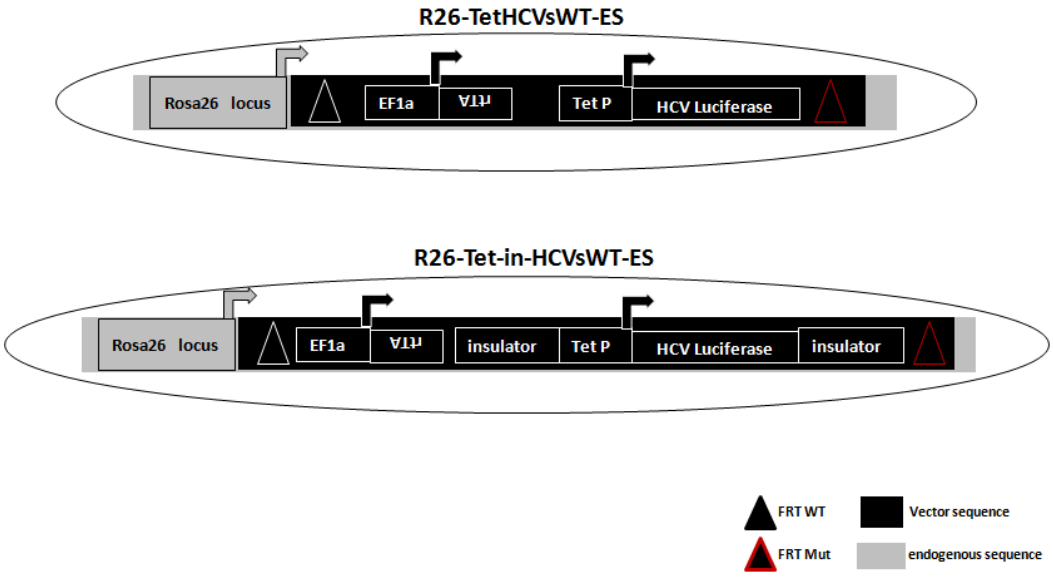
Moreover, one clone was identified (clone2') which showed higher basal expression but no induction. This phenotype is classified as profile 4.

Importantly, the expression phenotype of the four groups of clones was stable upon passaging for > 10 passages (data not shown).

Altogether, regulated expression could be achieved in various clones by flanking the Tet cassettes with two copies of the cHS4 insulator. However, the heterogenous expression in the various clones was unexpected since all the clones were genetically identical (isogenic) as a result of the targeted integration by RMCE. Still, a variation from clone to clone was observed. This suggests that random silencing processes occured during targeting and resulted in a

pronounced stochasticity. In conclusion, the cHS4 insulators can only partially overcome silencing.

a.)



b.)

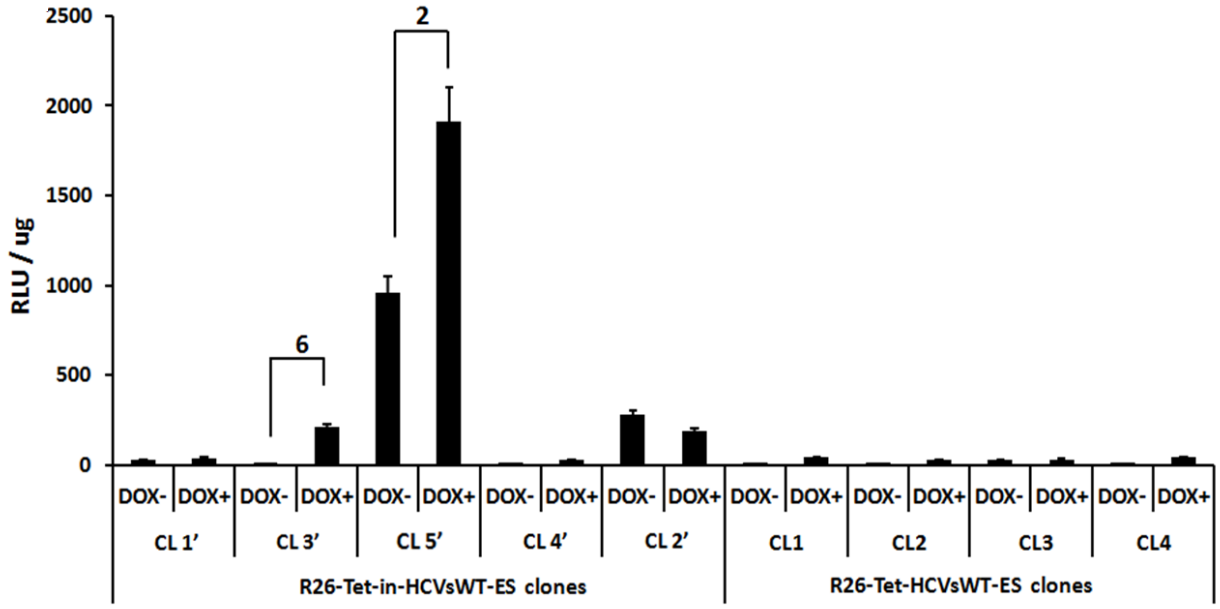


Figure 13: Tet-dependent luciferase expression level of ES clones within the Rosa locus with or without cHS4 insulator. a.) 2 copies of the cHS4 chromosomal insulator were introduced in TetHCVrsWT construct before the Tet promoter and after the HCV luc replicon. The insulated construct was then targeted into Rosa26 locus by RMCE and the R26-Tet-in-HCVsWT-ES cell clones were isolated. The design of the expression cassette is depicted. b.)

Individual R26-Tet-in-HCVsWT-ES clones were tested for luciferase expression after targeting. Five R26-Tet-in-HCVsWT-ES clones were selected. For comparison, four TetHCVrsWT-ES clones without insulators were analysed. 2.5µg/ml doxycycline was/was not administrated for 48h. rtTA was provided by transient transfection of pCMVRTA2HYG(P2288) 48 hours before the cells were harvested. Luciferase activity observed in relative light units (RLU) was normalized to µg of total protein present in the cell lysate. Error bars indicate standard deviation from 3 independent samples.

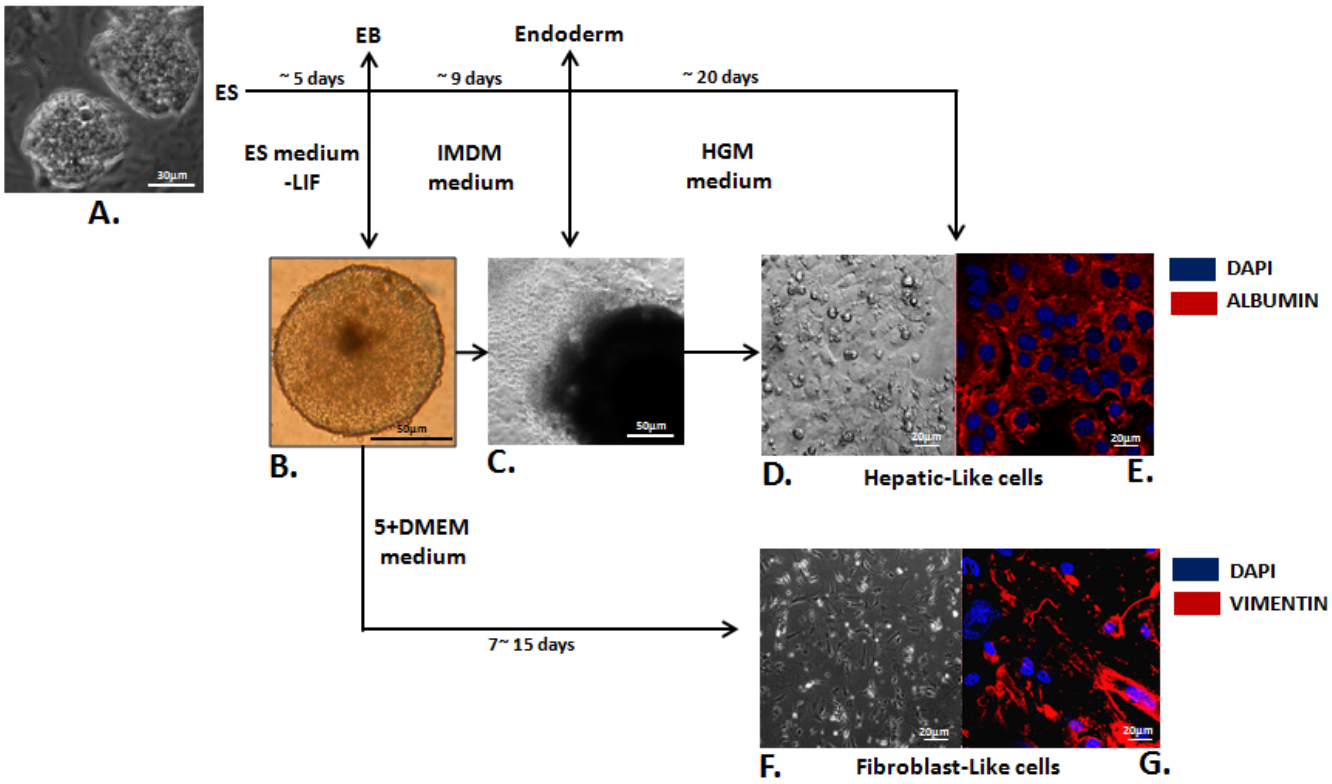
3.3.2 Expression stability of insulated cassettes upon differentiation

To investigate the stability of expression profiles of R26-Tet-in-HCVsWT-ES clones upon differentiation, the insulated clones R26-Tet-in-HCVsWT-ES clone 3' and 5' (representing profile 1 and 2, respectively) were differentiated to the hepatic lineage according to the protocol described in chapter 2.2.1.9. Representative pictures of the cells upon differentiation are shown in Figure 14a.

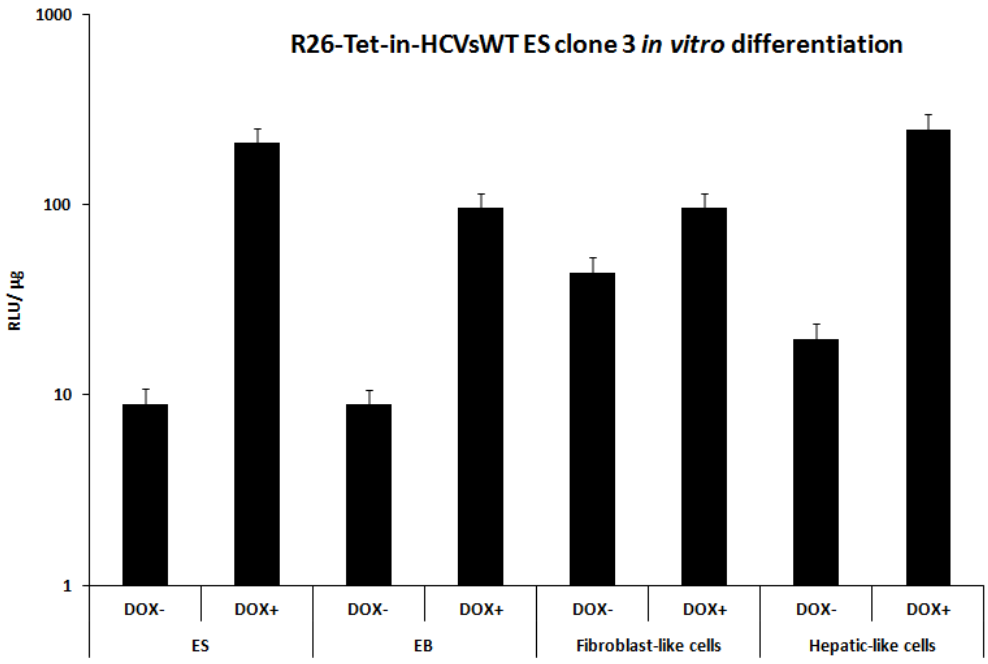
The expression status was again monitored at EB stage and upon differentiation to the hepatic lineage. Of note, irrespective of the differentiation stage (EB or hepatocyte-like stage), both of the insulated clones maintained the luciferase expression according to the profile presented in ES cells: in the hepatic state, clone 5' maintained a high basal level and low inducibility while clone 3' showed low basal level and high inducibility. In contrast, the non-insulated cells that were differentiated in parallel were silenced. This is in agreement with the previous data (chapter 3.3.1). Thus, in clones 3' and 5', the insulator elements could provide shielding of Tet cassette from silencing upon hepatic differentiation (Figure 14b and 14c).

To clarify whether the anti-silencing ability of cHS4 insulator was cell-type dependent the cells were also differentiated into fibroblasts. For this purpose, the embryonic bodies were seeded on un-gelatinized plates and cultivated in 5+ DMEM fibroblast medium (Figure 14a). Ten days later, the morphology of cells changed to fibroblastic phenotype(Togo et al., 2011). Cells were seeded in presence and absence of doxycycline and analyzed for luciferase expression. Interestingly, the two clones behaved differently. As demonstrated in Figure 14b-c, clone 3' kept luciferase expression upon fibroblast differentiation. In contrast, clone 5' showed a 10-fold reduction in luciferase expression upon fibroblast differentiation which was found to be associated with a reduced inducibility.

a.)


EB
Endoderm
ES
~ 5 days
~ 9 days
~ 20 days
ES medium
-LIF
IMDM
medium
HGM
medium
A.
B.
C.
D.
Hepatic-Like cells
E.
DAPI
ALBUMIN
5+DMEM
medium
7~ 15 days
F.
Fibroblast-Like cells
G.
DAPI
VIMENTIN

b.)


R26-Tet-in-HCVsWT ES clone 3 in vitro differentiation
RLU/ µg
1000
100
10
1
DOX-
DOX+
ES
EB
Fibroblast-like cells
Hepatic-like cells

c.)

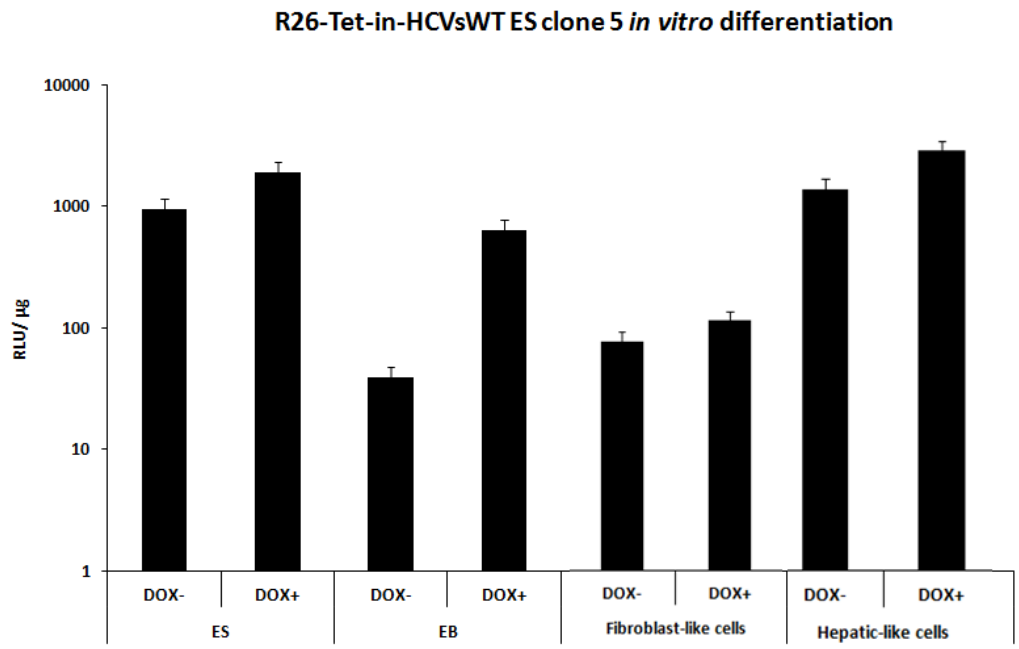


Figure 14: Expression pattern of the insulated Tet cassette in the Rosa26 locus upon *in vitro* differentiation.
a.) Cells targeted with insulated Tet cassettes as depicted in Figure 14 were subjected to *in vitro* hepatic differentiation as detailed in chapter 3.1.3. The hepatic phenotype was confirmed by both morphology and immunocytochemistry for albumin (Figure a-E, red fluorescence). For differentiation to fibroblasts, 25 EBs were seeded on an un-gelatinized well of a 6-well plate in 2 ml of 5+DMEM medium. 10 days after plating most of the cell differentiated spontaneously to fibroblast-like cells. Immuncytochemistry for vimentin was followed to confirm fibroblastoid state (red) (Figure a-G). Cells were maintained in presence/absence of 2.5 µg/ml Doxycycline during the whole procedure. rtTA was provided by transient transfection of pCMVRTA2HYG(P2288) two days before the cells were harvested. Luciferase activity observed in relative light units (RLU) was normalized to µg of total protein present in the cell lysate. Expression level of R26-Tet-in-HCVsWT-ES clone 3 (b) and R26-Tet-in-HCVsWT-ES clone 5 (c) upon differentiation to the various differentiation states is shown. Error bars indicate standard deviations from 3 independent samples.

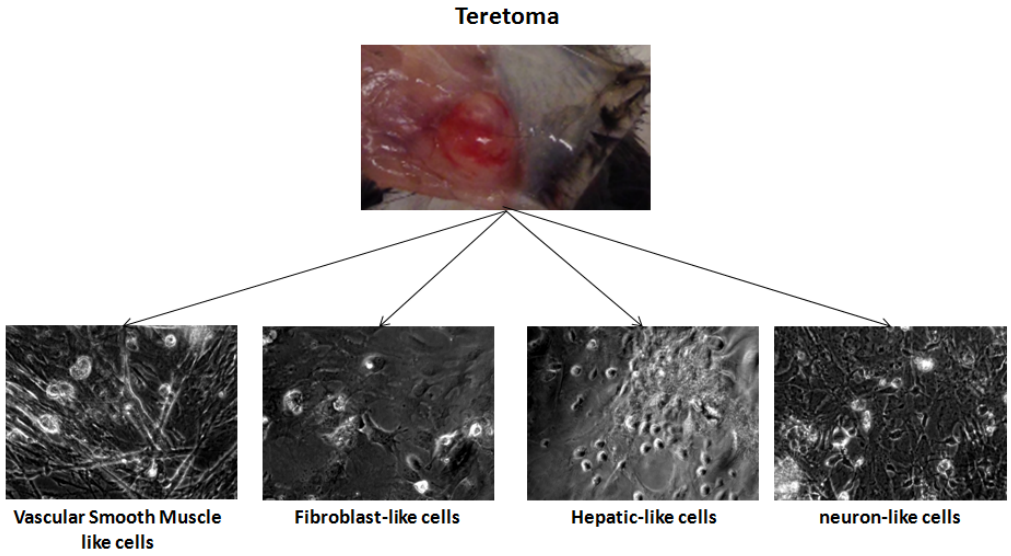
Since a change in regulated expression of the Tet cassette was not found upon *in vitro* differentiation into the hepatic but into the fibroblastic state, the cells were checked upon differentiation into the various tissues *in vivo*. For this purpose, clone3' and clone5' were subjected for teratoma formation upon subcutaneous injection. A teratoma is a kind of nonmalignant tumor that comprises a disorganized mixture of cells and small foci of tissue containing cells from all three germ-layers: ectoderm, mesoderm, and endoderm. This feature makes it a frequently used model to simulate embryonic development *in vivo*.

For teratoma formation, 10^6-10^7 cells of R26-Tet-in-HCVsWT-ES clone 3' or clone 5' ES cells were injected subcutaneously to the lower flanks of 8-12-week-old $RAG^{-/-}$ mice. 3-4 weeks after injection teratomas were visible. The mice were sacrificed to isolate teratomas. The teratomas were minced and cells were expanded *in vitro*. The cells were cultivated in presence/absence of doxycycline and transfected with pCMVRTA2HYG (Nr.2288) encoding for the transactivator rtTA. 48h later, the luciferase level was measured.

Interestingly, clone 3' maintained inducible luciferase expression at the stage of teratoma although the basal level slightly increased compared to the non-differentiated ES cell state. These results are in line with the inducible expression of luciferase upon *in vitro* differentiation. In contrast, expression in clone 5' was reduced to the basal expression level and could not be induced by doxycycline upon *in vivo* differentiation (Figure 15b).

To sum it up, cHS4 insulator could partially rescue Tet cassette expression in the Rosa26 locus in ES cells upon differentiation *in vitro* and *in vivo*. However, a pronounced clonal variation was observed. The data indicates that the cHS4 modification is not robust enough to protect the Tet promoter.

a.)



b.)

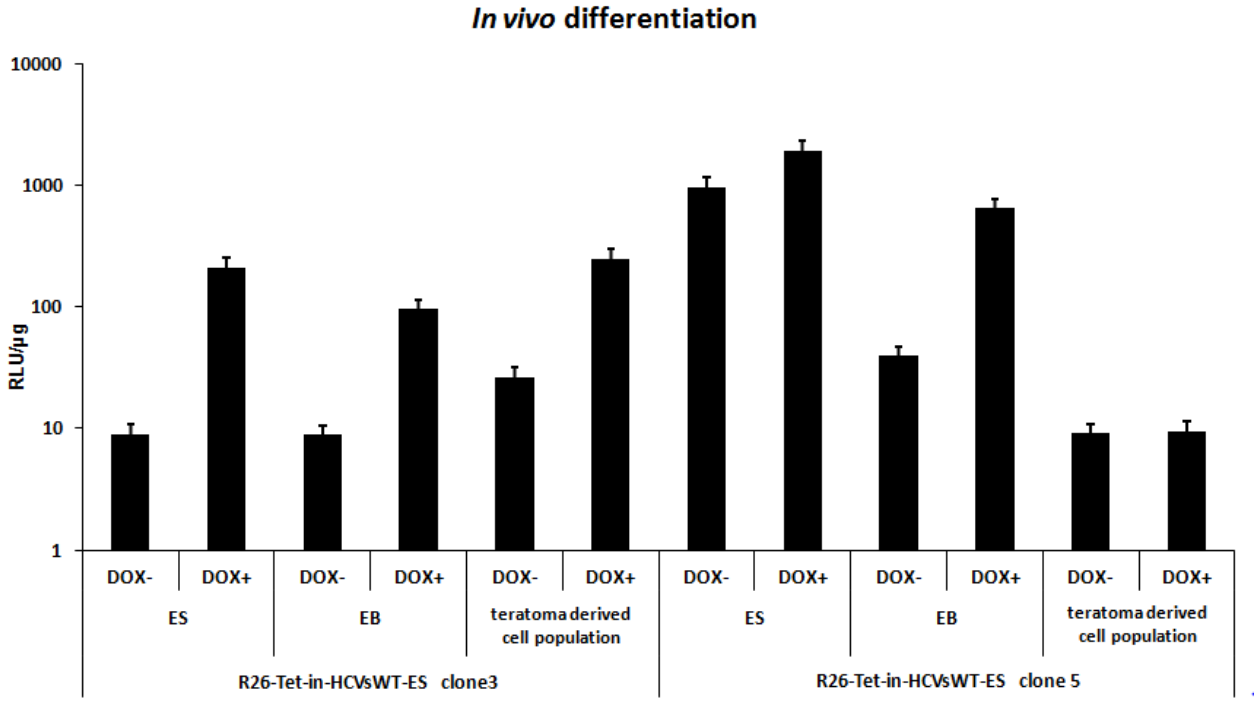


Figure 15: Expression level of the insulated Tet cassette in the Rosa26 locus in teratoma derived cells. a.) For *in vivo* differentiation, $1x10^6$-10^7/ 100 µl R26-Tet-in-HCVsWT-ES clone 3' or clone 5' were injected subcutaneously to the lower flanks of 8-12-week-old $RAG^{-/-}$ mice on the both sides, respectively. Teratomas were visible 4 weeks after injection and mice were sacrificed to isolate teratomas. Each teratoma was minced and was cultivated seperately for about 7 days on one well of a gelatinized 6 well plate. Different cell types were observed from the teratoma-derived cells as exemplified by the pictures (fibroblast-like cells, vascular smooth muscle like cell, hepatocyte-like cells and neuron-like cells are shown). b.) To evaluate expression, the cells were transfected with 2 µg rtTA expression plasmid pCMVRTA2HYG in the presence/absence of doxycycline. All the cells were harvested 48h after doxycycline treatment. Luciferase activity observed in relative light units (RLU) was normalized to µg of total protein present in the cell lysate. Error bars indicate standard deviations from 3 independent samples.

3.4 Targeted demethylation *in vitro* and *in vivo*

3.4.1 Targeted demethylation *in vitro*

The results of chapter 3.2 and 3.3 suggest that the Tet cassettes are prone to epigenetic silencing upon differentiation. The fact that expression could be at least partially rescued by Aza/cHS4 insulator suggests that silencing might be dependent on DNA methylation.

However, since neither the targeting into different chromosomal integration sites nor the flanking of the Tet cassette by cHS4 elements could fully/efficiently overcome the silencing of the cassettes, it was aimed at specifically modulating the epigenetic status of the Tet promoter by impairing or reducing its methylation.

For this purpose the TET methylcytosine dioxygenase 1 protein (TET1c) was exploited. This protein possesses enzymatic activity capable of hydroxylating 5mC to generate 5hmC which is then further processed and finally demethylated to unmethylated cytosine (chapter 1.2.1.2). To specifically target this enzymatic activity to the Tet promoter sequence, the catalytic domain of TET1 (TET1c) was fused to the bipartite rtTA, which encodes both a binding domain specifically for the operator sequences of the Tet promoter and an activation domain for active transcription (VP16). A tripartite fusion gene was cloned comprising the catalytic domains of TET1, the transactivating domain as well as the specific binding domain of rtTA (for details of the cloning see methods chapter 2.2.1.3). The vector encoding this tripartite fusion protein was designated as TET1c-rtTA and drives expression by the CMV promoter.

Further, a control plasmid encoding the truncated protein TET1c-U was established. TET1c-U carries only the catalytic domain of TET1c-U without any domain for specific DNA binding. The design of the vectors used in this study is shown in Figure 16.

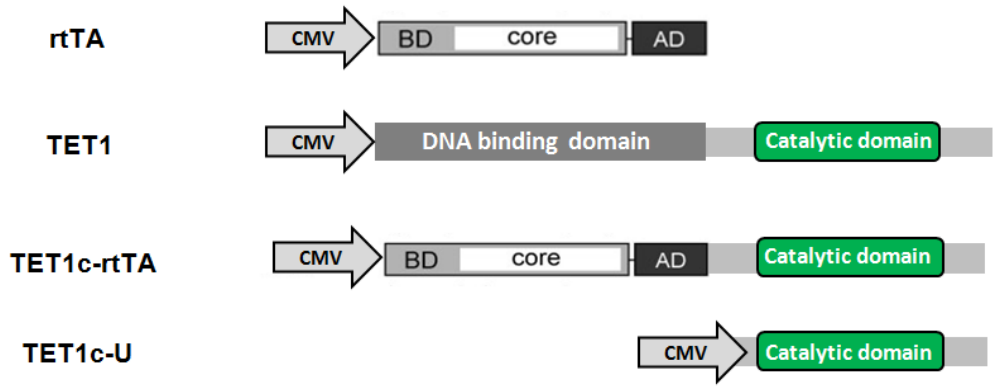


Figure 16: Schematic representation of the expression constructs for targeted demethylation. rtTA: This construct comprises the full length of bipartite rtTA including the domain (BD/core) for binding the operator regions

from Tet promoter as well as the activation domain (AD) for transcriptional activation. TET1: parental expression construct encoding the full length native TET1 comprising both the DNA binding domain and the catalytic domain. In the tripartite Tet1c-rtTA the rtTA was combined with the catalytic domain of TET1 (TET1c). TET1c-U: Tet1c-U has only with the catalytic domain of TET1 (TET1c). In all the vectors, expression of the respective genes is controlled by the CMV promoter.

First, the functionality of the transactivating domain of the tripartite fusion protein TET1c-rtTA was investigated. For this purpose, 293T cells were transiently transfected with a vector encoding TET1c-rtTA and the reporter plasmid Tetlucsr (Tet promoter driving luciferase expression). Upon 2 days in presence or absence of doxycycline the cells were harvested and analyzed for luciferase expression. As a positive control a plasmid carrying the unmodified transactivator (rtTA) was cotransfected with the same reporter plasmid under the same conditions.

As shown in Figure 17, co-expression of the tripartite fusion protein TET1c-rtTA could support doxycycline dependent expression the reporter. While in absence of doxycycline the expression levels were comparable, addition of doxycycline increased expression of the reporter. Together, these data indicate that TET1c-rtTA fusion protein can support Tet-inducible expression, confirming that the transactivator domain is not compromised in the tripartite fusion protein.

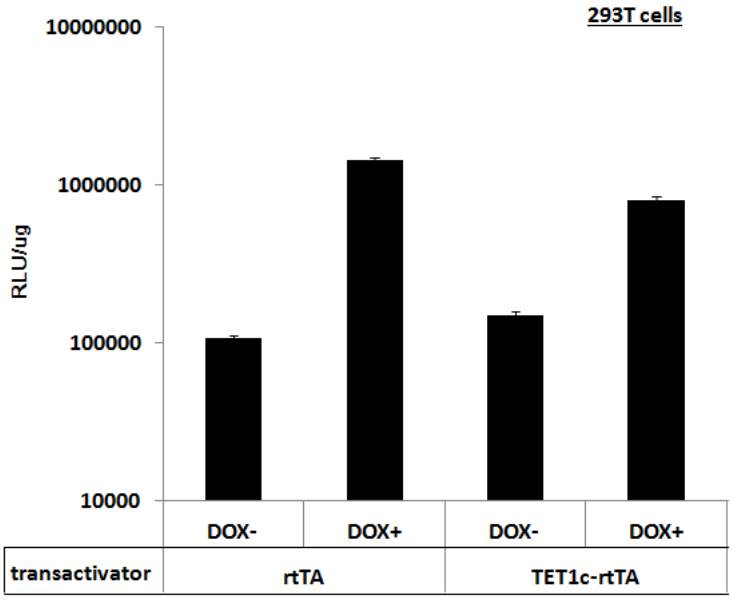
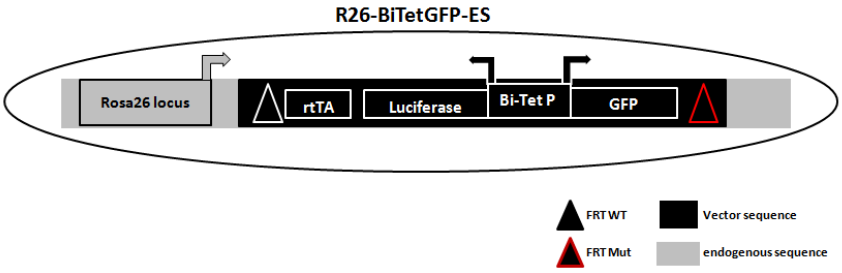


Figure 17: Transactivation potential of TET1c-rtTA in 293T cells. 24 hours after seeding, $1x10^5$ HEK 293T cells were seeded per 6 well, transient co-transfections were conducted. 1 µg Tetlucsr luciferase expression reporter plasmid was cotransfected with either 1 µg rtTA expression (pCMVRTA2HYG,) vector or 1 µg of TET1c-rtTA. Cells were harvested 48 hours after 2.5 µg/ml doxycycline treatment. Luciferase activity of cell lysates was measured. Relative light units (RLU) were normalized to µg of total protein present in the cell lysate. Error bars indicate standard deviation from 3 independent samples.

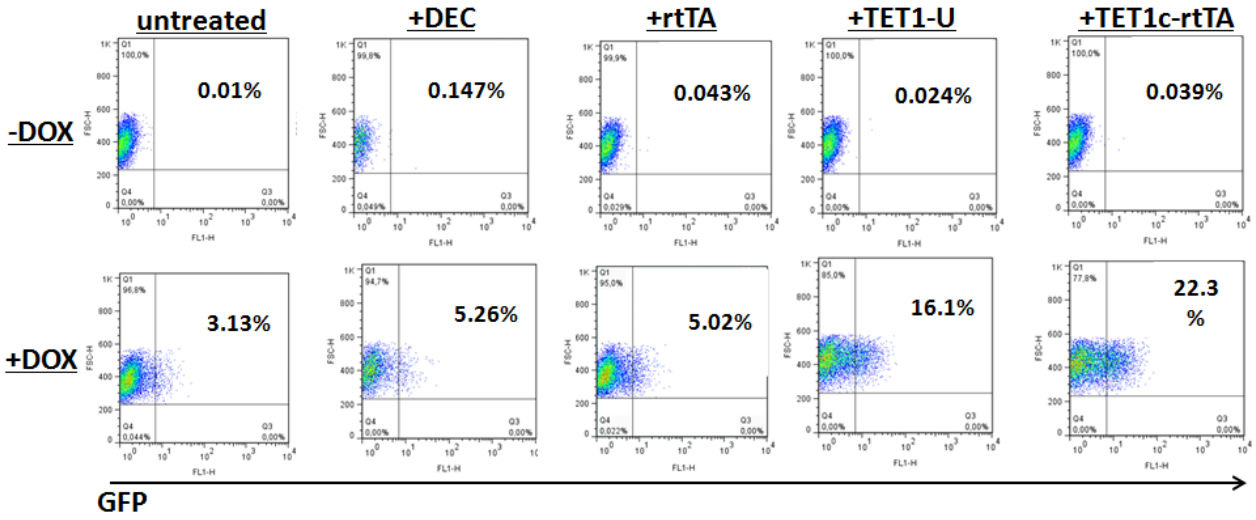
To evaluate the demethylation capacity of TET1c-rtTA, it was evaluated if the tripartite fusion protein can reactivate silenced Tet cassettes. To this end, R26-BiTetGFP ES cells were analyzed for GFP expression upon transient transfection of the TET1c-rtTA plasmid. rtTA was transfected as a control. Further, Tet1c-U was transfected which lacks a specific DNA binding domain. As a positive control, the cells were treated with the chemical inhibitor of methylation decitabine (5-aza-2'-deoxycytidine). All cells were incubated with or without doxycycline for 72 hours.

In agreement with the data shown in chapter 3.1.2, the low numbers of GFP expressing R26-BiTetGFP ES cells only increased slightly in presence of DNMT inhibitor – in this case Decitabine. However, a pronounced increase of GFP expression was observed upon transfection of the tripartite fusion gene TET1c-rtTA. A population of GFP positive cells (22%) was observed in the TET1c-rtTA transfected sample while the basal expression was not significantly higher compared to the other analyzed samples (see Figure 18b for representative samples and Figure 18c for the summary of 3 independent samples per clone). Of note, transfection of rtTA alone did not increase the number of expressing cells dramatically. These data show that TET1c-rtTA can clearlyactivate Tet cassette in the Rosa 26 locus. This suggests that the demethylation domain can improve expression in these cells. Interestingly, also the TET1c-U transfected cells showed a clear GFP fraction in the induced state of around 16%. This suggests that a certain reactivation of Tet driven GFP was achieved also in these ES cell clones.

a.)


R26-BiTetGFP-ES
Rosa26 locus
rtTA
Luciferase
Bi-Tet P
GFP
FRT WT
FRT Mut
Vector sequence
endogenous sequence

b.)


untreated
+DEC
+rtTA
+TET1-U
+TET1c-rtTA
-DOX
+DOX
0.01%
0.147%
0.043%
0.024%
0.039%
3.13%
5.26%
5.02%
16.1%
22.3 %
GFP

c.)

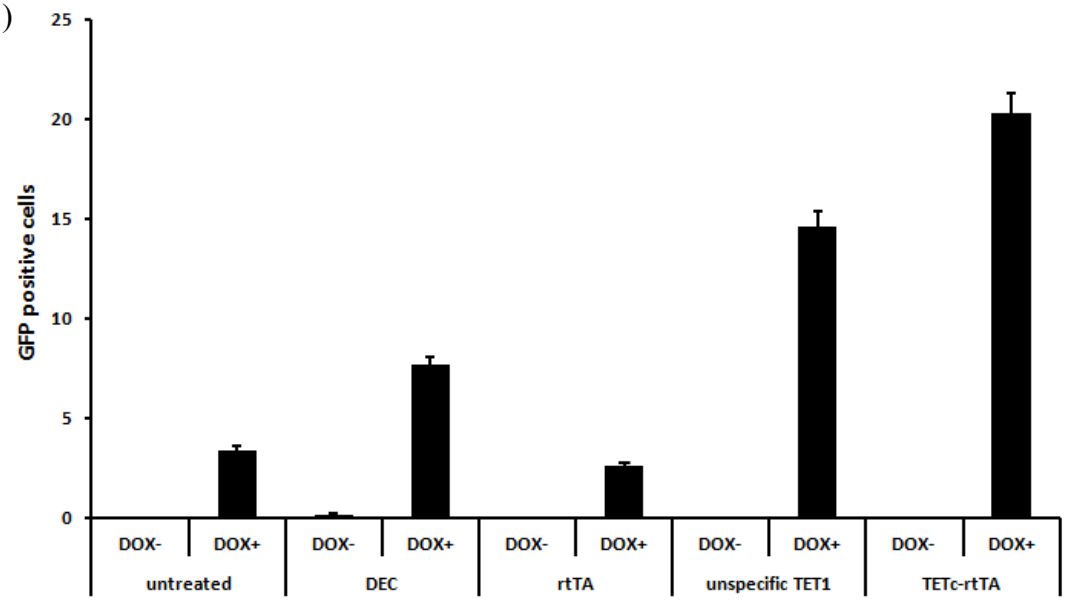
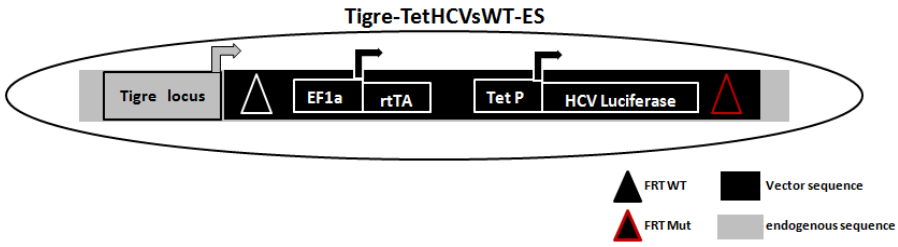
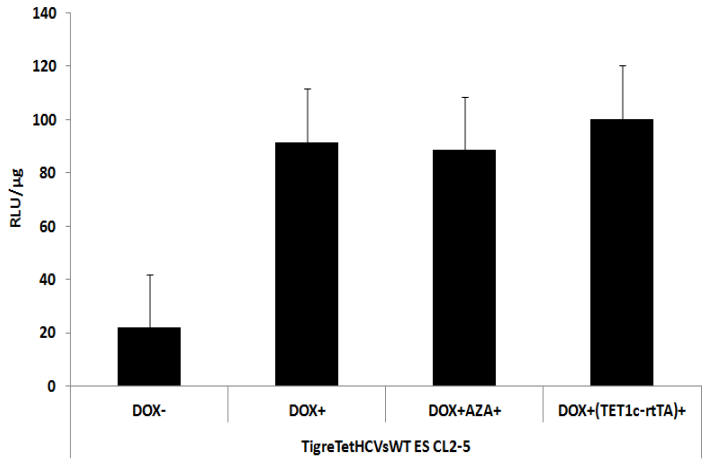

GFP positive cells
25
20
15
10
5
0
DOX-
DOX+
untreated
DEC
rtTA
unspecific TET1
TETc-rtTA

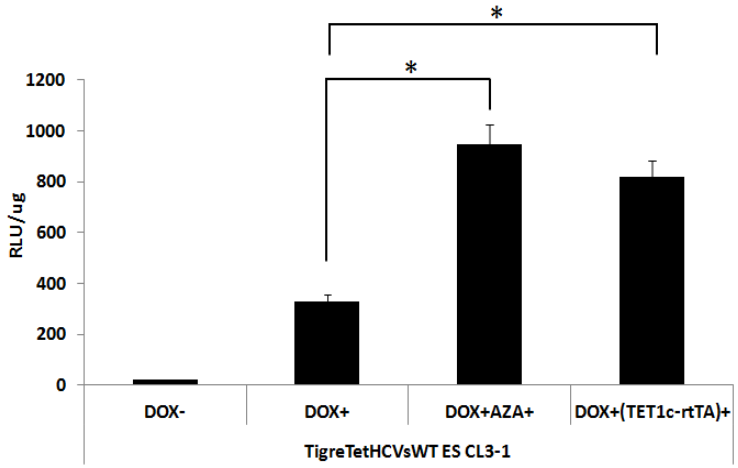
Figure 18: TET1c-rtTA induced GFP reactivation in ES cells in Rosa26 locus. a.) Depiction of the Tet construct within R26-BiTetGFP ES cell. This cell line encodes a bi-directional Tet promoter driving GFP and luciferase in the Rosa26 locus while the transactivator rtTA is provided by the endogenous Rosa26 promoter. b.-c.)1 x10^5 R26-BiTetGFP ES clone7 cells per 6 well were seeded on irradiated mouse embryonic fibroblast feeder cells. 48 hours after seeding ES cells were treated as follows. Untreated: non-transfected cells; + DEC: incubation with 0.5 µM decitibine for 48 hours; rtTA: transfection with 2 µg rtTA expression vector (pCMVRTA2HYG); Tet-1U: transfection with 2µg TET1c-U; TETc-rtTA transfection with 2µg TET1c-rtTA plasmid. Samples were cultivated for 72 hours in presence or absence of doxycycline as indicated. GFP positive cells were analyzed by flow cytometry and data were analyzed by FLOWJO (FLOWJO; LLC). Representative FACS plots are shown in b) and mean expression from 3 independent samples is presented in c). Error bars indicate standard deviation from three samples.

It was investigated if the construct could also rescue the transgene expression in the Tigre locus. Thus, TigreR26-TetHCVsWT-ES cell clone 2-5 and clone 3-1, as well as TigreTetlucs ES clone 4-1, and clone 1-3 (as described in chapter 3.1.3) were transiently transfected with the TET1c-rtTA or treated with the demethylating agent Aza according to the procedure described above. Aza treatment could re-activate expression only in the TigreR26-TetHCVsWT-ES clone 3-1 and TigreTetlucs ES clone 1-3 while in the other clones expression was not changed by Aza. Interestingly, transfection of Tet1c-rtTA similarly showed clear expression of GFP in the same two clones while no reactivation could be achieved in the other two. Indeed, all clones which could react to Aza showed also an increased expression of luciferase upon TET1c-rtTA transfection, while other clones could not react to both (Figure 19a-b). Thus, this suggests that Aza as well as TET1c-rtTA mediated reversion of silencing can only be achieved in certain cells/cell states while others are resistant to these treatments.

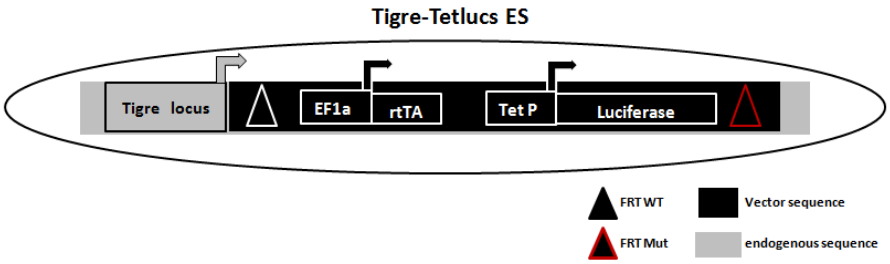
a.)




140
120
100
80
60
40
20
0
RLU/µg
DOX-
DOX+
DOX+AZA+
DOX+(TET1c-rtTA)+
TigreTetHCVsWT ES CL2-5


*
*
1200
1000
800
600
400
200
0
RLU/ug
DOX-
DOX+
DOX+AZA+
DOX+(TET1c-rtTA)+
TigreTetHCVsWT ES CL3-1

b.)


Tigre-Tetlucs ES
Tigre locus
EF1a
rtTA
Tet P
Luciferase
FRT WT
FRT Mut
Vector sequence
endogenous sequence

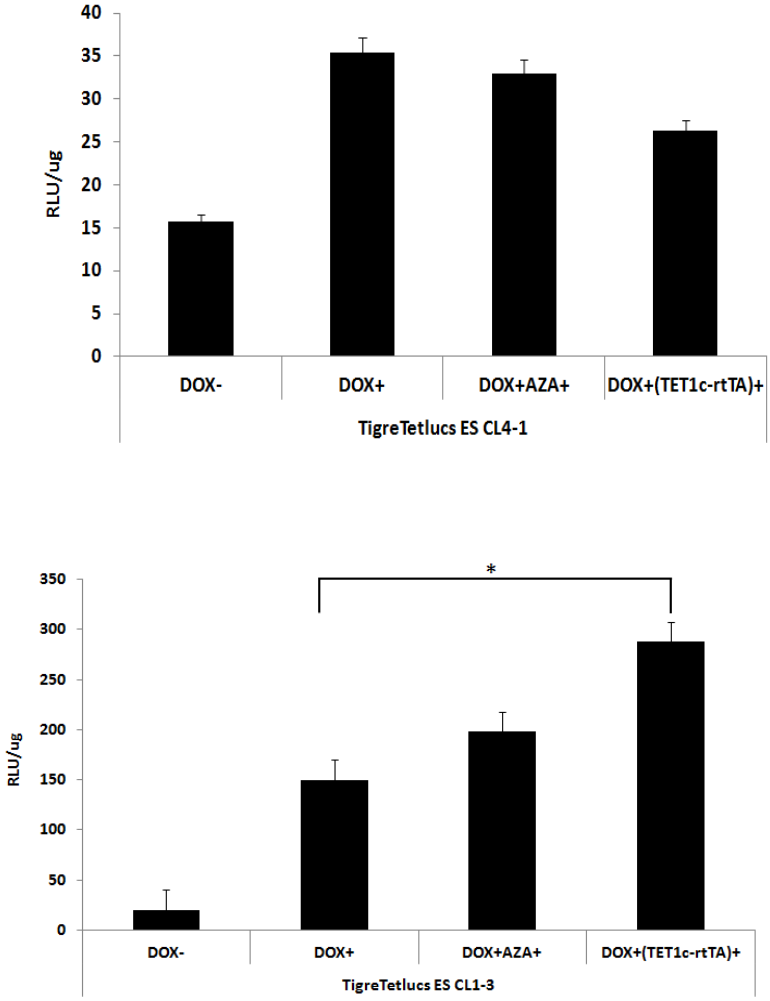


Figure 19: TET1c-rtTA induced reactivation of luciferase in ES cells in the Tigre locus. Tigre-Tetlucs ES clone4-1 and 1-3 as well as Tigre-TetHCVsWT-ES clone 2-5 and 3-1 were used in this experiment. The design of the constructs is indicated. Tigre-TetHCVsWT-ES encode the HCV luc reporter while Tigre-Tetlucs ES cells have the Tetluc reporter. Both constructs carry the Tet promoter driving luciferase in Tigre locus while the transactivator rtTA was expressed from the EF1α promoter from the same targeting construct. 1 x10^5 cells of each clone were seeded on the irradiated mouse embryonic fibroblast feeder cells in gelatinized 6 wells. 48 hours after seeding the ES cells they were treated with the following conditions: DOX-: inoculated without Doxycycline for 72 hours; DOX+: inoculated with 2.5 µg/ml doxycycline for 72 hours; DOX+AZA+: inoculated with 2.5 µg/ml doxycycline and 1 µM/ml Aza for 72 hours; DOX+ (TET1c-rtTA)+: transfected with 2 µg TET1c-rtTA plasmid transiently and incubated with doxycycline for 72 hours. Luciferase activity observed in relative light units (RLU) was normalized to µg of total protein present in the cell lysate. Error bars indicate standard deviation from 3 independent samples and significance was calculated using online *P* value calculator.

Finally, also the COL1A1 locus were analyzed for the responsivenes to TET1c-rtTA. However, for this locus only ES cell clones were isolated that were resistant to Aza treatment. In agreement with the observation for the Tigre locus, all the clones also failed to respond to TET1c-rtTA (data not shown).

To sum it up, rescuing of the Tet cassette expression by TET1c-rtTA could be observed in two different loci (Rosa26 and Tigre) and could be demonstrated for several cassette designs. Interestingly, some clones did not respond. Of note, the non-responding clones were also resistant to chemical activation by Aza. This might indicate that the states of silencing are different in these clones. For a more detailed discussion of this observation please see chapter 4.3.1.

3.4.2 Targeted demethylation *in vivo*

3.4.2.1 Targeted demethylation in vivo in R26-BiTetGFP mice upon TET1c-rtTA treatment

The *in vitro* investigation of the tripartite TET1c-rtTA fusion protein showed efficient rescue of the Tet cassette expression in targeted ES cells which was comparable to or even better than the treatment with chemical demethylation reagents (Figure 18-19). To investigate the role of the potential of TET1c-rtTA fusion protein *in vivo,* this protein was expressed in several transgenic animals. Since it was shown that the R26-BiTetGFP ES cell clone could be reactivated by the Tet1c rtTA construct *in vitro* the corresponding R26-BiTetGFP transgenic mouse line was used (see Figure 20a for cassette design).

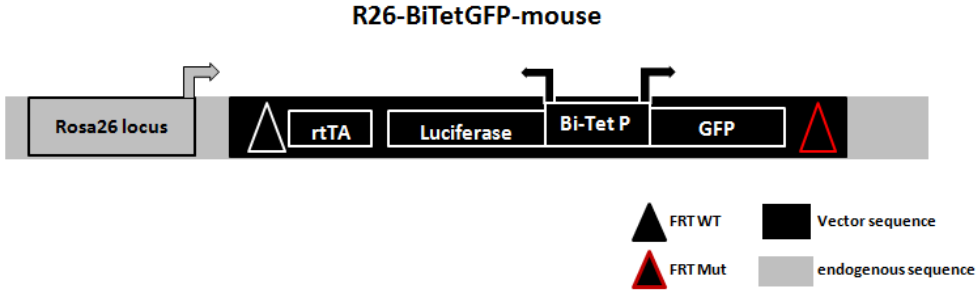
To deliver the TET1c-rtTA plasmids to the mouse, the plasmids were injected by hydrodynamic tail vein (HDTV) injection. This method gives rise to gene transfer preferentially in the liver of the mice (chapter 2.2.1.3). Briefly, 25 µg of the plasmid DNA was diluted in 0.1% NaCl at the volume of 10% of the body weight of the animal. This volume was injected within 10 seconds into the tail vein. Injection was done both in untreated mice and mice preconditioned by a 7 day feeding with doxycycline. Luciferase expression was monitored by *in vivo* bioluminescence imaging using the IVIS machine.

TET1c-rtTA, the rtTA, the TET1c-U as well as a plasmid free PBS control were hydrodynamically injected. The results of the expression analysis 36 hours after injection are shown in Figure 20c. Of note, TET1c-rtTA injected mice exhibited a remarkable increase of luminescence signal. This increase in expression was only obtained in the doxycycline induced group of animals but not in the un-induced animals (data not shown). In contrast, none of the injections with rtTA, TET1c-U or PBS as controls showed significant rise of luciferase expression regardless of doxycycline induction. This suggests that TET1c-rtTA, but not the unspecific control TET1c-U or the rtTA alone can reactivate expression.

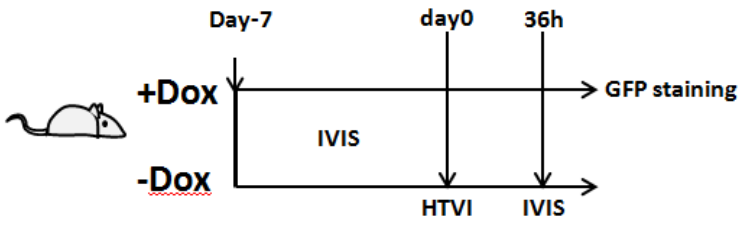
To evaluate whether the liver cells homogenously or heterogeneously activated transgene expression, the specific increase in transgene expression was analyzed by histology on cellular level. Therefore, 36 h after HDTV injection/delivery of the plasmid mice were sacrified and liver samples were collected analyzed for GFP expression. In the liver of non-treated or mock-treated animals, only very few GFP positive cells were identified (about 1 cell per view or 0.89% of cells). A clearly increased number of GFP positive cells were observed in TET1c-rtTA mice. Quantification by counting the number of GFP positive cells per view revealed that about 16 GFP positive cells per view corresponding to 16% of hepatocytes showed GFP expression. In contrast, no obvious difference could be observed in rtTA or PBS injected groups (Figure 20d).

These data indicate that both GFP and luciferase were reactivated upon HDTV injection of the tripartite TET1c-rtTA plasmid. Of note, neither TET1c-U nor rtTA could rescue the silenced Tet promoter inducible expression of luciferase.

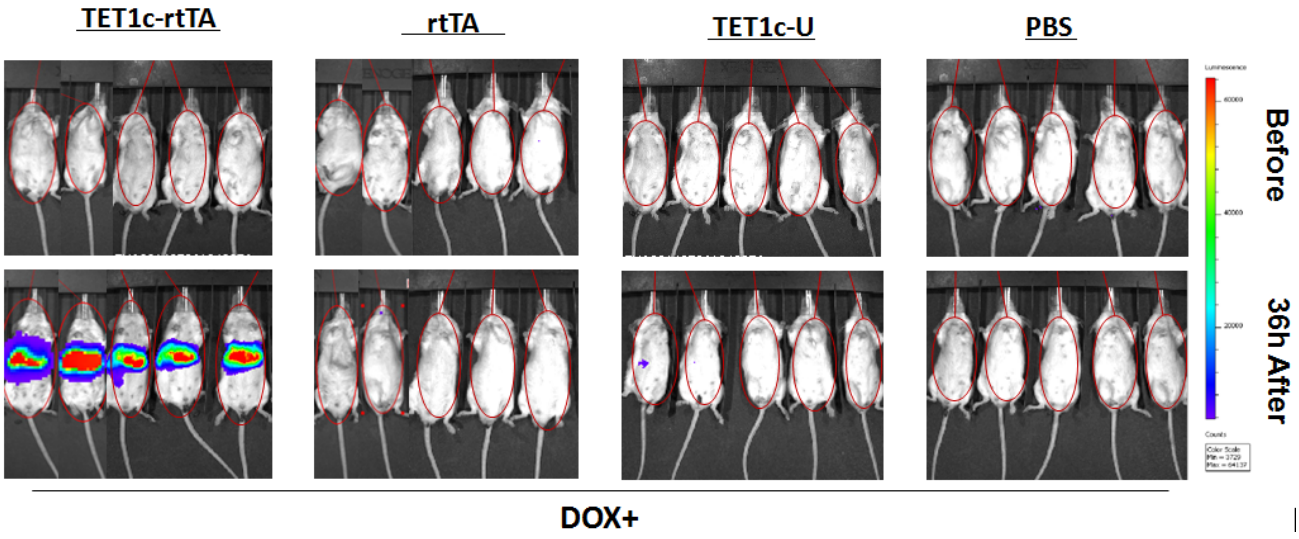
a.)



b.)



c.)



d.)

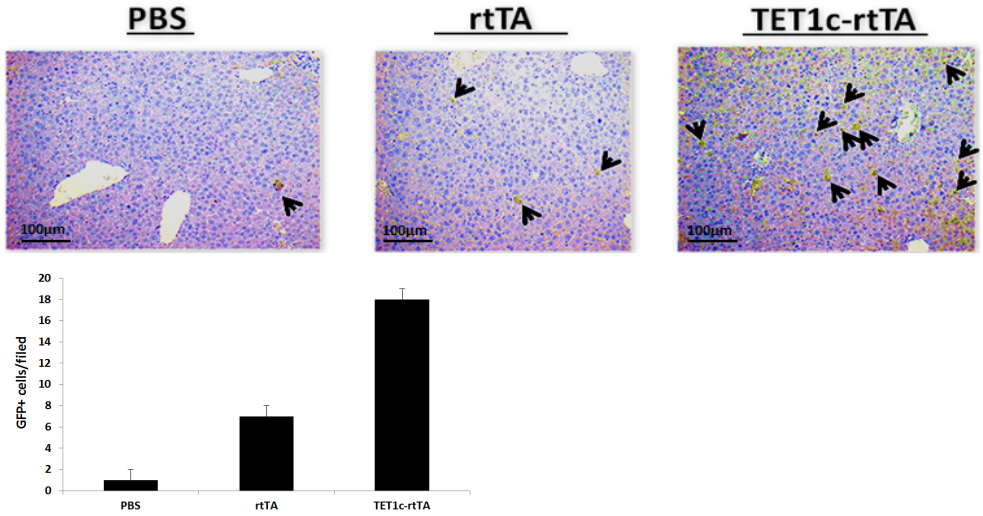
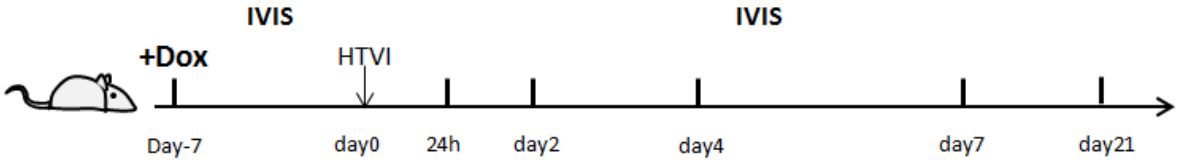


Figure 20: TET1c-rtTA reactivates Tet driving GFP and luciferase expression *in vivo*. a.) Depiction of the targeting construct integrated into the R26-BiTetGFP mice. b.) Workflow of the treatments of the transgenic mice. 2 mg/ml doxycycline was applied via the drinking water; 5 mice per group were injected with the following plasmids as indicated: TET1c-rtTA; PBS; rtTA; TET1-U. c.) *In vivo* imaging of the mice 36 hours after injection. d.) GFP was detected by specific antibody staining of the liver tissues from mice injected with PBS, rtTA and TET1c-rtTA. For quantification, at least 3 samples were included for each mouse and GFP positive cells were counted in one field. Error bars indicate standard deviation from 3 independent samples.

3.4.2.2 HDTV-mediated reactivation of expression in vivo in R26-BiTetGFP mice is transient

HDTV mediated transfer of plasmids leads to transient expression due to the episomal state of transduced vectors (Liu, Gan, Yang, Zhang, & Sun, 2014; Peng et al., 2015). Thus, this experimental setting allows investigating whether TET1c-rtTA would enable a long-term reactivation of luciferase expression that is maintained even in absence of the initial trigger. Thus, the experiment described in the previous chapter was repeated and the expression of luciferase was analyzed at day 2, day 4, day 7 and day 21 after injection. TET1c-rtTA dependent activation of expression could be observed on day 2 and 4. Interestingly, it was found that luciferase expression vanished again about 7 days post injection (Figure 21b). This suggests that the reversion of silencing is transient, probably due to the transient expression of HDTV mediated expression of the TET1c-rtTA plasmid. Thus, a single administration of TET1c-rtTA is not sufficient to keep the Tet promoter in an active state.

a.)



b.)

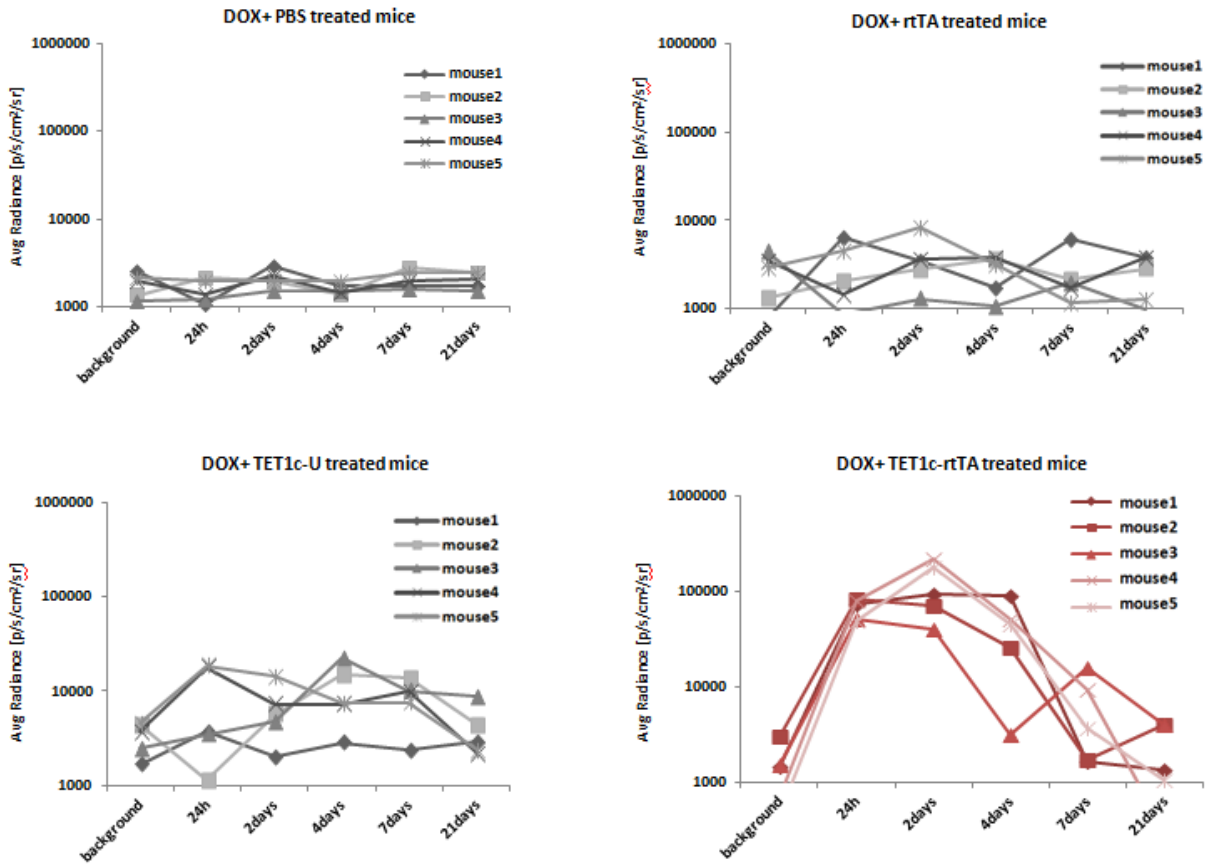


Figure 21: Kinetics of TET1c-rtTA reactivates Tet promoter driving GFP and luciferase expression *in vivo*. a.) Workflow of the treatment of the transgenic mice. 2 mg/ml doxycycline was used applied in the drinking water; 5 mice per group were injected with the following plasmids: TET1c-rtTA; PBS; rtTA; TET1-U. b.) Kinetics of the hydrodynamically injected R26-BiTetGFP mice measured in the indicated mice the IVIS over 21 days.

3.4.2.3 Regulation of doxycycline controlled expression in R26-BiTetGFP mice upon TET1c-rtTA treatment

In the previous experiments the expression levels upon TET1c-rtTA expression were evaluated in mice pretreated with doxycycline. An obvous increase of luciferase and GFP expression was observed. However, these experiments do not allow the evaluation of the doxycycline dependent activation of transgene expression in individual mice. Thus, to evaluate the regulation capacity an alternative experimental setting was used. This time, all the mice were injected by HDTV in the non-induced state, i.e. without being treated with doxycycline. No increase in luciferase expression could be observed 24 hours after injection compared to day 0 as expected (Figure 22). Then, the injected animals were induced with doxycycline and analyzed on day2.

Upon doxycycline treatment all the injected animals showed an increase in luciferase expression. This shows that expression critically depends on the presence of doxycycline and confirms the regulation capacity of the TET1c-rtTA construct in the injected animals (Figure 22).

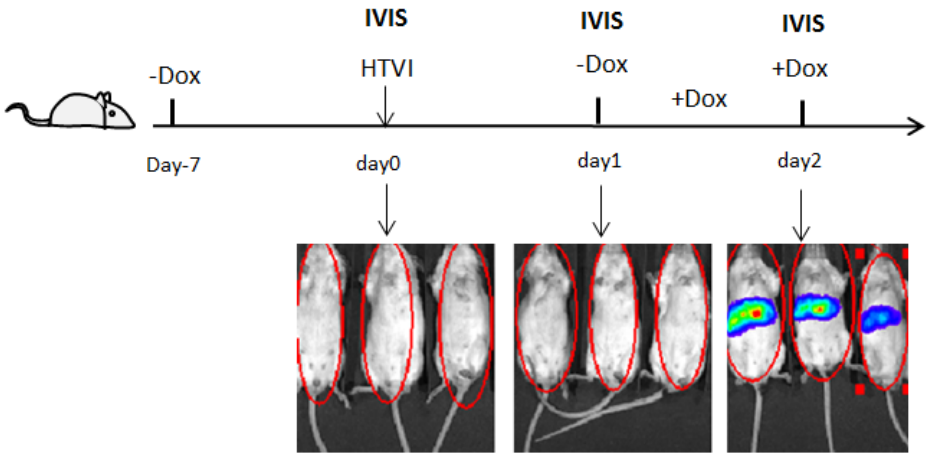


Figure 22: TET1c-rtTA supports regulation of Tet-driven luciferase expression. Schematic representation of the experimental set up: 25 µg plasmid was injected to the tail vein as specified in Figure 21. One day after the injection the animals were measured in the IVIS. After the first measurements the animals were induced with 2 mg/ml doxycycline which was applied in the drinking water. 24h after beginning of the doxycycline feeding the animals were measured again in the IVIS.

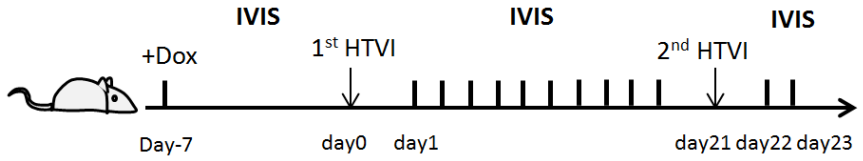
3.4.2.4 Re-administration of TET1c-rtTA leads to re-induction of luciferase expression

Finally, it was tested if the TET1c-rtTA mediated activation of luciferase expression could be repeated upon a second HDTV injection of the TET1c-rtTA. For this purpose, two cycles of HDTV injections were performed and the animals were analyzed according to the experimental schedule depicted in Figure 23a.

After injection, a transient expression of luciferase was observed which lasted for 2-7 days. After 21 days, the signal was completely abrogated and a second injection was applied. The luciferase levels were found to be highly increased, which was comparable to the first application of TET1c-rtTA. Thus, a reactivation of the Tet promoter in the R26-BiTetGFP mice resulted in a

similar expression of the construct. This confirms the conclusion that the TET1c-rtTA needs to be continuously present to reactivate the Tet promoter *in vivo*.

a.)



b.)

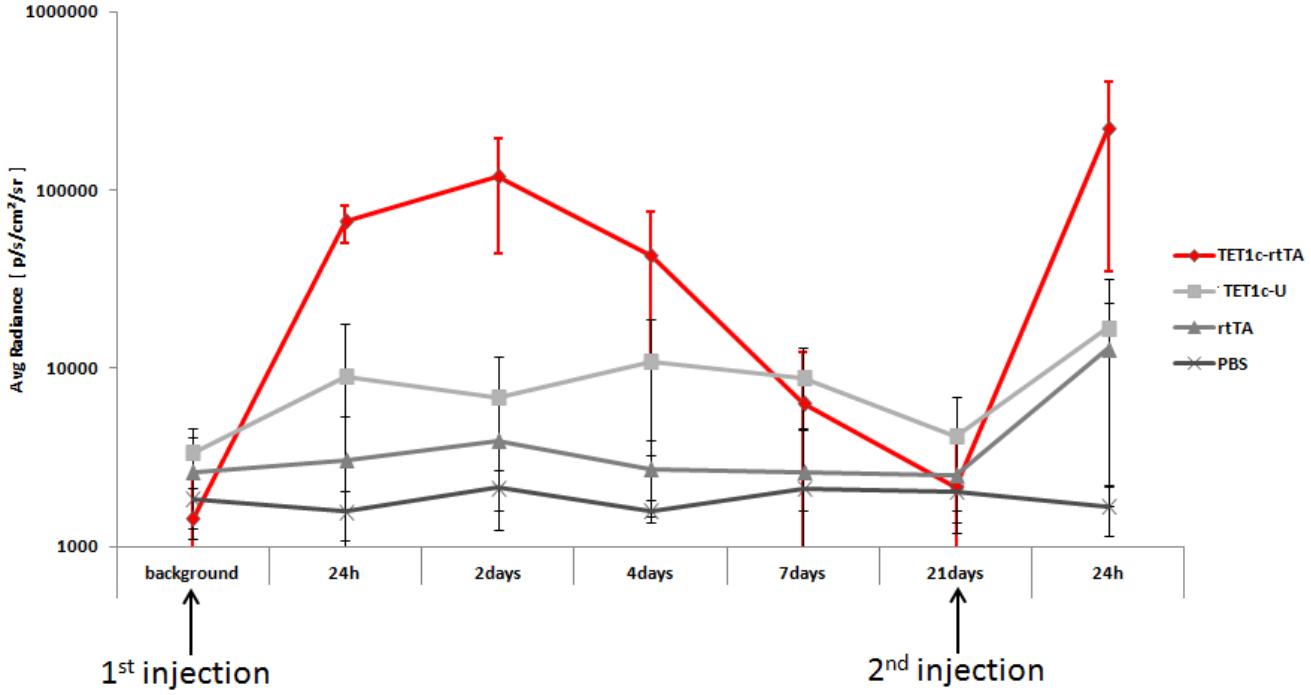


Figure 23: Repeated administration of plasmids by HDTV. a.) Schematic depiction of the experimental set up: the R26-BiTetGFP mice were fed with 2 mg/ml doxycycline and HDTV was performed. Luciferase was monitored by *in vivo* imaging. The injection of the indicated plasmid was repeated after 21 days while the luciferase expression was analysed on day 23 after the first injection. b.) Kinetics of the luciferase expression in groups of 5 mice was measured by bioluminescence. Error bars indicate standard deviation in these groups of mice.

3.4.2.5 Targeted demethylation in vivo in LUC8.10 mice upon TET1c-rtTA treatment

To further confirm the general capacity of the TET1c-rtTA in reactivation of the silenced Tet cassettes another transgenic mouse line was investigated. This mouse carrys a Tet-controlled

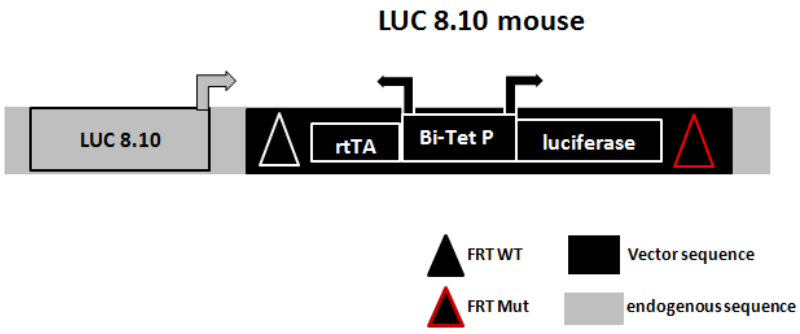
luciferase reporter cassette in a random integration site designated as 8.10 locus (Figure 24a). Previous data in our lab exhibited heterogeneous luciferase expression in this mouse model (Riemer, Thesis). Only 38 out of 78 mice (49%) showed luciferase expression and 62% out of the luciferase expressing mice displayed regulated luciferase expression by doxycycline trearment.

It was tested if the lack of expression in the 8.10 locus could be overcome by TET1c-rtTA. Thus, non-luciferase-expressing luc8.10 transgenic mice were selected for the experiment and subjected to HDTV injection of the TET1c-rtTA plasmid. Briefly, LUC 8.10 mice were initially induced with doxycycline for 7 days. Then the hydrodynamic tail vein injections of TET1c-rtTA, rtTA, PBS were conducted, respectively. The animals were analyzed every day in the first week and afterwards at day 9,11,21,29 and 42 in the IVIS.

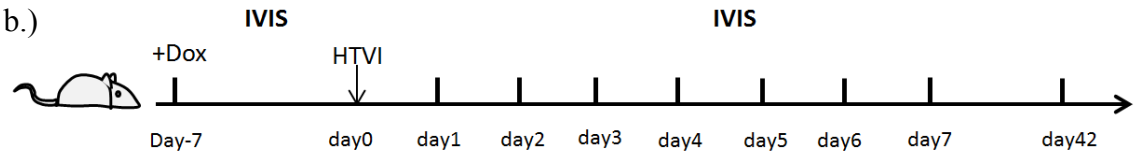
As shown in Figure 24c, none of the animals expressed luciferase before the application of the plasmids by HDTV. However, the luciferase signal of the TET1c-rtTA injected animals was significantly increased over a period of at least 29 days. Overall, the TET1c-rtTA injected group exhibited high induction (82-fold). In contrast the induction-fold of the rtTA controls groups were much lower (only 12-folds) (Figure 24d).

To investigate the kinetics for the TET1c rtTA injected mice, the animals were analyzed over long time. The signal was found to be dropped down to the background levels after 42 days. At this time point, re-injection was performed to evaluate whether a reactivation of luciferase expression can be achieved. Similar to the reactivation pattern observed in TET1c-rtTA treated R26-BiTetGFP mice, the expression peak of luciferase in the LUC 8.10 mice was achieved upon 24h-48h and disappeared 7 days post injection (data not shown here).

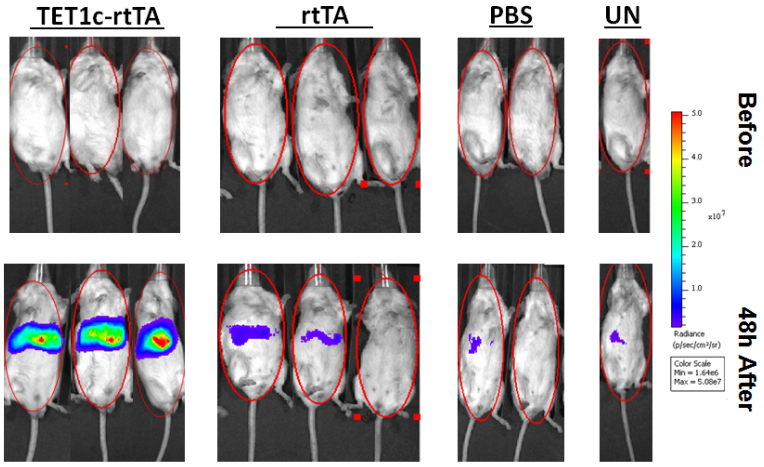
a.)



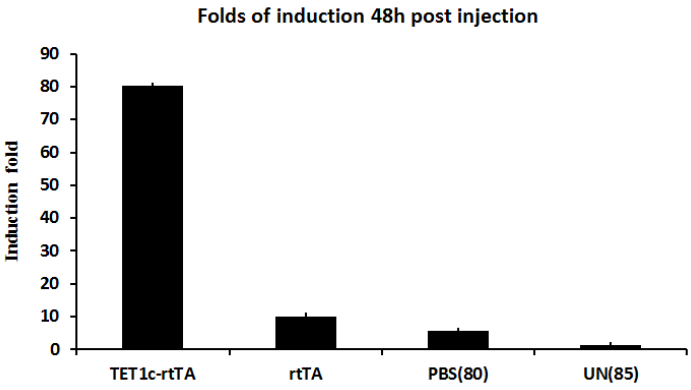
b.)


IVIS
IVIS
+Dox
HTVI
Day-7
day0
day1
day2
day3
day4
day5
day6
day7
day42

c.)


TET1c-rtTA
rtTA
PBS
UN
Before
48h After
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.64e6
Max = 5.08e7

d.)


Folds of induction 48h post injection
Induction fold
90
80
70
60
50
40
30
20
10
0
TET1c-rtTA
rtTA
PBS(80)
UN(85)

e.)

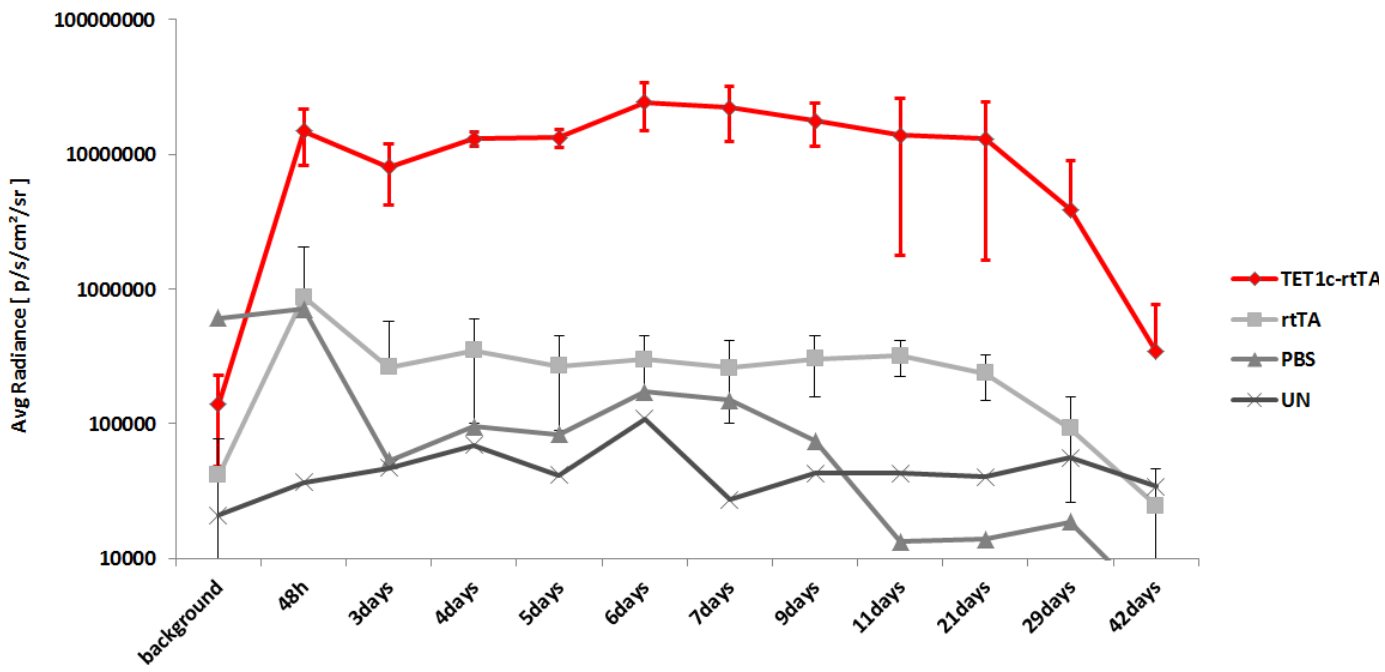


Figure24. TET1c-rtTA reactivates Tet driven luciferase expression in the 8.10 locus. a.) HDTV injection in LUC8.10 mice. b.) Workflow of the treatment of the transgenic mice. Doxycycline administration and HDTV injection was done as described in Figure 24a. Each group contained at least 3 mice (7-14 week old mice were chosen). The constructs used for injections are indicated as untreated group (UN) (c.) *In vivo* imaging of the mice 48 hours after injection. (d.) Fold-induction of each group 48 hours post injection. Error bars indicate standard deviation from 3 independent samples. (e.) The time course of luciferase expression in luc8.10 mice upon HDTV injection with the indicated vectors.

In conclusion, a reactivation of the Tet cassette by episomal expression of TET1c-rtTA could be confirmed in an independent chromosomal integration site.

4. DISCUSSION

The tetracycline inducible system (Tet system) has been widely used in mammalian cell lines and transgenic animal models by the virtue of its convenience and inducibility (Ed, 2000; Shockett & Schatz, 1996). However, although the system has been successfully exploited for many applications, there are also reports showing that the performance of the Tet system can be limited by epigenetic silencing resulting in severe loss of transgene expression (Pankiewicz, Karlen, Imhof, & Mermod, 2005; Takiguchi et al., 2013; Zhu et al., 2007a). Unfortunately, the experimental settings in the various studies (such as cassette design, readout, and chromosomal integration site) were quite different. Thus, general conclusions are hard to extract from literature which limits the rational improvement of the Tet system. The aim of this work was to systematically compare the performance of the same Tet cassette in defined genomic loci and develop strategies to overcome the silencing of the Tet cassette in cells and mice.

In this study, several strategies have been utilized to achieve this aim. On the one hand, the performance of the Tet cassettes was evaluated at three previously identified chromosomal integration sites (Rosa26 locus, COL1A1 locus and the Tigre locus). Results show that in ES cells the Tigre locus provided the best expression of the Tet cassette. However, none of the tested loci supported the Tet cassette expression in mice. On the other hand, cHS4 chromosomal insulators were introduced into the Tet cassette to protect it from the influence of the genomic context. However, this exhibited only partial rescuing of the Tet cassette expression *in vitro* and *in vivo*. A novel strategy was developed which was based on site specific/targeted active demethylation of the Tet cassette by application of the methylcytosine dioxygenase subunit of TET1. This strategy could be shown to provide efficient activation of the silenced Tet cassettes both in cells and in mice in different loci, thereby overcoming the loss of expression.

4.1 Silencing of the Tet promoter: impact of the CMV promoter element

An integral part of the classical Tet promoter is a minimal CMV promoter (chapter 1.1.4.2). The minimal CMV promoter is the streamlined version of CMV which only comprises the basic elements required for initiation of mammalian transcription. The native CMV (cytomegalovirus) promoter is routinely used for transient and stable transgene expression in various cell systems

and also for the production of proteins in biotechnology (Davies et al., 2011). However, it has been reported increasingly that this virus-derived element also can be silenced *in vitro* and *in vivo* (Everett et al., 2004; Morita, Arii, Christensen, Votteler, & Sundquist, 2012; Spencer, Gugliotta, Koenitzer, Hauser, & Wirth, 2015). For instance, Brooks and his colleagues injected rats with an Adenovirus containing the human fibroblast growth factor 4 (hFGF-4) gene driven by the cytomegalovirus (CMV) promoter and enhancer (CMV- PE). Methylation of the CMV-PE in the muscle at both CpG and non-CpG sites was observed 24 hours after virus administration and was increased at day 7 days after injection (Brooks et al., 2004). This kind of DNA methylation induced CMV silencing has been suggested to be a cellular defense mechanism against the transcription of foreign genes (Doerfler, 1992). The silencing of the CMV promoter in case of a single copy insertion in the Rosa26 locus has also been observed. Tchorz and his colleagues characterized several ubiquitous promoters (pCAG, EF1a and CMV) in the modified Rosa26 locus (the endogenous Rosa26 locus has been silenced) in the mice. They demonstrated that the ubiquitous pCAG promoter in the modified Rosa26 locus offered highest transgene expression, while CMV promoter did not support ubiquitous EGFP expression in the mice. The transgene expression could only be detected in testis, but not in salivary glands, heart, pancreas, liver, kidney, fat, muscle, brain and testis (Tchorz et al., 2012a). These reports are in contrast to the reports that are showing the CMV promoter could support long and stable transgene expression (F. Li, Vijayasankaran, Shen, Kiss, & Amanullah, 2014). This indicates that the silencing of the CMV promoter is also dependent on the nature of the chromosomal integration sites. Accordingly, screening for suitable sites is one frequently used method to circumvent silencing.

The Tet promoter is widely used for inducible transgene expression *in vitro* and *in vivo* (Stieger, Belbellaa, Le Guiner, Moullier, & Rolling, 2009; Sun, Chen, & Xiao, 2007; Takahashi, Watanabe, Nakagawa, Kawakami, & Sato, 2008; Wang et al., 2013). However, in recent years the silencing of Tet promoter has also been shown.

Wörtge and colleagues reported a mouse model carrying the Tet promoter integrated into the Rosa26 locus - a well described chromosomal site that has been shown to support ubiquitous expression of transgenes both from the endogenous Rosa26 promoter as well as upon integration of heterologous promoters (Zambrowicz et al., 1997). Interestingly, upon integration of the Tet promoter into the Rosa26 locus the mice showed very sparse doxycycline-activated expression of

different reporter genes in the brain, mosaic expression in peripheral tissues, and more prominent expression in erythroid, myeloid and lymphoid lineages, in hematopoietic stem and progenitor cells, and in olfactory neurons (Wörtge et al., 2010a). Furthermore, Peixin Zhu also reported in 2007 the silencing of the Tet promoter in neurons in a mouse model generated upon random integration of the transgene cassette. They discovered that the Tet promoter became functionally silenced in the majority of neurons when the Tet promoter was inactive during development (Zhu et al. 2007b). Likewise, Pankiewicz and his colleagues observed the silencing of the Tet cassette upon random integration into the mouse genome. They found that the silenced transgene integrated into a poorly accessible chromatin structure (Pankiewicz et al., 2005).

A similar phenomenon was observed in the transgenic ES and mouse lines established in our lab upon targeting the Rosa26 locus with Tet cassettes. Even in a clonal population of targeted ES cells, none of the cell lines with various cassette designs showed homogeneous transgene activation in individual cells (Kruse, Wirth et al., unpublished results). Moreover, only part of the transgenic littermates expressed the transgene while others could not give rise to any gene expression (Thesis Kruse, 2013). The methylation profile of the targeted Tet promoter was analyzed upon sorting for low and high expressing ES cells. A high CpG methylation ratio in the Tet promoter was discovered in low/non expressers while in contrast expressers contained significant lower methylation ratio (Thesis Spencer, 2014). These results indicate that the Tet promoter is prone to be silenced by DNA methylation which correlates to the lack of expression. Silencing can occur at various sites and even upon the integration into an active transcribed locus.

In this study, the Tet cassette with the same setting was introduced to different loci by Flp recombinase-based RMCE. Two loci, COL1A1 and Tigre locus were used which were previously discussed to support regulated expression (Beard et al., 2006b; Premsrirut et al., 2011; Zeng et al., 2008). Interestingly, in this study, these reports could not be confirmed. Rather, the analysis showed that the Tet cassettes were found to be partially silenced at COL1A1 and Tigre locus in ES cells, and totally silenced in mice.

The actual mechanisms associated with transgene silencing are not fully understood. Various factors have been shown to contribute, including the nature of integration sites, transgene copy number (D W Emery et al., 2000; Kong et al., 2009), methylation of the promoter regions (Meyer, 2013), deacetylation of histones (Oyer, Chu, Brar, & Turker, 2009) and repeated sequences within the transgene cassettes (Garrick et al., 1998).

In this thesis the RMCE targeting strategy resulted in single copy integration of cassette and thus could eliminate the copy number influence. On the one hand, the silencing of the Tet cassette upon differentiation could be overcome by DNMT inhibitors in the Tigre locus of ES cells (chapter 3.2), which indicated that the block of DNA methylation would protect transgenes from silencing. On the other hand, the active demethylation was successfully achieved by recruiting the dioxygenase domain of TET1 to the silenced Tet promoter in ES cells and mice (chapter 3.4). These results implied that the Tet promoter methylation was associated with transgenes silencing also in the other two loci and the silencing could be overcome.

4.2 Towards a predictable transgene expression an transgenic animals

A predictable expression of transgenes is important for a transgenic models study and antibody production. Basically, predictable expression is a consequence of two variables: the site of integration and the design of an expression cassette.

4.2.1 Safety of the characterized genomic loci for the targeted transgenesis

There are some well-characterized genomic loci, which have been reported to support predictable and reproducible transgene expression whilethey exhibited no detectable pathological disturbance to the endogenous genes. For instance, mouse models are established which rely on the integration of the transgene into the Rosa26 locus and are highly predictable in terms of transgene expression (Casola, 2010). However, also various systems with heterologous promoters are used - with variable success. Recently, a systematic comparison in ES cells showed that the Rosa26 locus does not support expression of all promoters to the same degree (Tchorz et al. 2012).

As for the Tet promoter in the Rosa26 locus, unstable and heterogeneous expression of transgenes was observed. Haenebalcke generated an inducible mouse model which carried the Tet promoter driving vascular endothelial growth factor isoform 164 (VEGF164) in the Rosa26 locus (EGFP-luciferase was included as reporters). They observed a decline of VEGF164 expression in transgenic ES cells 11 days after culturing. Furthermore, different transgene

expression levels were detected in different organs in the transgenic mice established from these cells (Haenebalcke et al., 2013).

Heterogeneous expression of the Tet promoter was also observed in the Rosa26 locus in this thesis. R26-BiTetGFP ES cells, which carry a bi-directional Tet promoter driving GFP and luciferase, exhibited low luciferase expression. When the cells were analyzed for GFP, only 3% R26-BiTetGFP ES cells showed a GFP signal, which indicated that only few cells of the isogenic population expressed while the others were completely silenced. This result suggests that the promoter was not homogenously inactivated but was inactivated in the majority of cells and thus overall expression is low. This might suggest the Tet promoters in different cells are undergoing stochastic processes, which might govern the status of the promoter.

Of note, the silencing of the Tet promoter occurred regardless of the direction of transcription relative to the Rosa26 endogenous promoter (uni-direction or bi-direction) or introduced transgenes (luciferase or luc-HCV) (Figure 9-10). These results indicate that the silencing of the Tet promoters does not depend on different transgenes or cassettes design.

However, silencing of the Tet promoter seems to be highly dynamic. Wan and his colleagues established CMV-GFP mice that ubiquitously express the rtTA from the Rosa26 locus and also carry a Tet-GFP cassette inserted into the COL1A1 locus as a single-copy gene. They found that fetal doxycycline exposure/rtTA activation led to silencing of the COL1A1 integrated Tet-GFP cassette in adult mice. They further gave evidence that the inheritance of epigenetic perturbation was dependent on the timing of the perturbation as well as the location of the perturbation (Wan et al., 2013).

In this thesis, the silencing of the Tet cassette was also observed in the loci other than the Rosa26 locus: COL1A1 locus and Tigre locus in transgenic mice. As shown in 3.1.1, although the Tet-luciferase/GFP expression could be detected in ES cell state to a certain extent upon *in vitro/ in vivo* differentiation no Tet-luciferase/GFP expression could be detected at all. This implies that differentiation during embryonic development might also contribute to the silencing of Tet promoter.

These facts indicate that even the well characterized genomic loci could hardly guarantee transgene expression *in vitro* and *in vivo*. The expression fashions of transgenes depends on targeting constructs, targeted transgenes (Figure 9-10), the types of the cells (Zhu et al., 2007a) and even the stages of the embryonic development (Wörtge et al., 2010a).

4.2.2 Improve the targeting vector by introducing chromatin modifiers

The silencing of the Tet promoter was proposed to be overcome by introducing proper chromosomal elements that could shield the constructs from negative influences of the integration site. These chromosomal modifiers could protect the constructs either by keeping the chromatin open and/or by protecting the transgene cassettes against negative effects like heterochromatin spreading (Nair, Jinger, & Hermiston, 2011; Quilici et al., 2013; N. Uchida, Hanawa, Yamamoto, & Shimada, 2013; F. Zhang et al., 2010b).

A chromatin insulator is one of the described chromosomal modifiers. It is a DNA sequence which has been shown to protect a target gene from the position effects exerted by neighboring domains (Burgess-Beusse et al., 2002; Recillas-Targa et al., 2002). The cHS4 insulator, which was used in this thesis, is a 1.2 kb DNA element originally derived from the 5' regulatory region of the chicken β-globin locus (Chung, J H). Previous reports have shown that by insertion of one or two copies of the 5'HS4 full length elements or only the core elements, one could protect the transgene from silencing (Bell, West, and Felsenfeld 1999). cHS4 insulators have also been used to optimize the Tet cassette. Shi reported in 2011 that the cHS4 element could improve Tet cassettes upon random integration as achieved by lentiviral gene transfer. This resulted in a better transgene expression compared to the expression without using insulator (Shi et al., 2014).

However, high variability in the expression of cHS4 modified Tet cassettes was observed in this thesis. Although elevated luciferase expression was observed in some cHS4 modified Tet cassette targeted ES clones, also clones were identified which showed no improvement at all (Figure 14). This was unexpected since the clones were generated upon RMCE and can be considered to be genetically identical (isogenic). These data indicate that cHS4 chromatin insulator could not robustly shield the Tet cassette in the Rosa 26 locus in targeted ES clones. These results are in accordance with the report published by Grajevskaja in 2013. They observed that the cHS4 insulator failed to shield the thenkx2.5 promoter from position effects in zebrafish (Grajevskaja, Balciuniene, & Balciunas, 2013). In their study, they introduced 1.2 kb cHS4 insulators to a RFP expression cassette driven by lens-specific gamma-crystalline promoter in the zebrafish genome. They found that flanking a transgenic construct by cHS4 insulation sequences lead to overall increase in the expression of RFP. However, a very high degree of variability of

RFP expression with respect to embryos was also observed, which indicated cHS4 insulators fail to fully protect transgene expression in this setting.

The failure of cHS4 insulator in fully protecting the Tet cassette is not clear. The reasons could be that the construct setting in this thesis was different from the literature which fulfilled robust protection of transgenes by cHS4 insulators, or the dysfunction of cHS4 in the specific locus or in combination with the Tet promoter.

4.3 Rescue of the transgene expression by targeted demethylation

Naturally, DNA demethylation happens during the mammalian development and other biological processes like sperm/ eggs reprogramming after fertilization (Gu et al., 2011). It is the direct removal of a methyl group independently of the DNA replication. The mechanism of the natural active DNA demethylation has been unclear for a long time. However, in 2009, Zhang and his colleagues showed that Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) protein possesses enzymatic activity capable of hydroxylating 5-methylcytosine (5mC) to generate 5-hydroxymethylcytosine (5hmC) by oxidation of 5mC in an iron and α-ketoglutarate dependent manner. This conversion of 5mC to 5hmC has been proposed as the initial step of active DNA demethylation in mammals (Wu & Zhang, 2011). The TET1 protein is composed of a methylated CpG (MeCpG) binding domain and a catylatic domain (H. Zhang et al., 2010). The separated MeCpG binding domain and the catalytic domain of TET proteins provide the possibility of redirecting the protein to specific loci of choice.

DNA demethylation could also be achieved un-naturally. For years researchers utilized Aza to inhibit DNMTs during the synthesis of new DNA strands. In this case the methylation of the new DNA strands would not occur. Therefore, the blocking of DNMTs is an induced, replication-dependent and non-natural process. However, their toxicity resulted from the global impact on the whole genome and replication-dependent demethylation (because DNMT inhibitors could only inhibit methyl group transfer on newly synthesized DNA) pattern still largely limited their practical applications (Jüttermann, Li, & Jaenisch, 1994).

4.3.1 Response variability to TET1c-rtTA within treated mice

In this study, TET1 was successfully utilized for targeted demethylation by fusing the catalytic domain of TET1 with the transactivator rtTA, which specifically binds the Tet promoter (TET1c-rtTA). Reactivation of the Tet cassettes in different loci in ES cells and mice was achieved by the overexpression of a plasmid encoding TET1c-rtTA. In the ES cells carrying a silenced Tet cassette in the Tigre locus or in Rosa26, transient transfection of TET1c-rtTA led to an increase of the transgene expression (Figure18-19). This demonstrates the reactivation in different integration loci and different construct settings. In silenced mice, 50-120 -fold improvement of transgene expression could be detected 36-48 hours after delivery of the plasmid via HDTV injection, which indicates that the reactivation of the Tet cassettes could even be achieved in the mice (Figure20-21).

However, among all TET1c-rtTA injected R26-biTetGFP mice, 2 failed to show an elevated luciferase signal. This did not correlate with the level of background expression level since the expression differences among the mice prior to injection were not significant (data not shown). Interestingly, these none-responders could neither be reactivated by Aza treatment. A similar impairment to reactivate the expression was also observed in the tested silenced ES clones. For instance, the luciferase signal of the Tigre-TetHCVsWT ES clone 3-1 and the Tigre-Tetlucs ES clone 1-3 increased pronouncedly after both TET1c-rtTA and Aza treatment. Nevertheless, the stable silencers like Tigre-TetHCVsWT ES clone 2-5 and Tigre-Tetlucs ES clone 4-1 responded neither to the treatments (Figure 19). These data suggest that there might be different states of epigenetic silencing and only some of these states can be reversed by demethylation. Furthermore, the silencing states might be different even within a clonal population of cells (Figure 18-19).

Epigenetic repression of gene expression might be divided into different phases characterized by different combinations of DNA/histone modifications, such as DNA methylation, histone acetylation / methylation / phosphorylation / ubiquitination / sumoylation (Chahwan, Wontakal, & Roa, 2010; Dhar, Vishal, Sharma, & Kaul, 2014; Gaál & Oláh, 2014; Matzke & Mette, 2000; Ptashne, 2007). These phases might accumulate or self-reinforce over time (Lachner et al., 2001). As proposed in Figure 25, when the chromatin is in a fully active state and all the activation markers bind, transcription is ongoing. However, once the ultimate silencing state is achieved (all the silencing collaborators are recruited on the promoter), the chromatin is so condensed that a solid and stable repression of the promoter is achieved. Such a state will be sustained and hard

to change. However, also intermediate states could exist in which the chromatin state stays in between of these two extreme states. In such an intermediate state, the chromatin is plastic that could easily be converted by epigenetic modifiers like Aza or - like in this study – by enzymatic demethylation of the promoter.

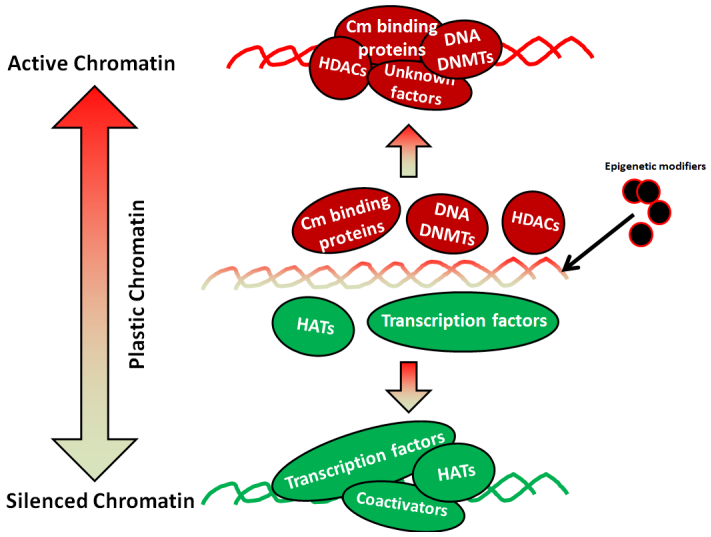


Figure 25. The transition from the active chromatin to the silent chromatin. The chromatin is considered to have three states: active, intermediate and silenced. In the intermediate phase chromatin is plastic and dynamic and can easily be converted by epigenetic modifiers (e.g. demethylateon). When all the silencing collaborators team up on the promoter, the ultimate silencing phase is achieved and the repression of the promoter can not be reverted. HAT - Histone acetyltransferase; DNMTs - DNA methyltransferase; HDACs - Histone deacetylase; Cm - methylated cytosine.

During the silencing process, the relationship between the two major epigenetic modifications - DNA methylation and repressive histone modifications, i.e. the dynamics of the modification, is still unclear until now (François Fuks, 2005). On the one hand, some scientists proved that DNA methylation is followed by repressive histone modifications. One example is represented by Lehnertz and coworkers who observed that loss of H3K9 methylation in histone-lysine N-methyltransferase SUV39H1 ES cells could decrease Dnmt3b-dependent CpG methylation at major centromeric satellites in mammals (Lehnertz et al., 2003).

On the other hand, there is also evidence that DNA methylation is a prerequisite for repressive histone modifications. For example, Fuks and coworkers found that the methylated CpG

dinucleotides recruited MeCP2 (methyl CpG binding protein 2), which furthermore recruited histone deacetylase (HDAC) to the methylated DNA. This results in chromatin remodeling and reinforce the silencing status *in vivo* (Francois Fuks et al., 2003). Thus, they suggested a model for a self-reinforcing epigenetic cycle that might strengthen a repressed chromatin state (Figure 26).

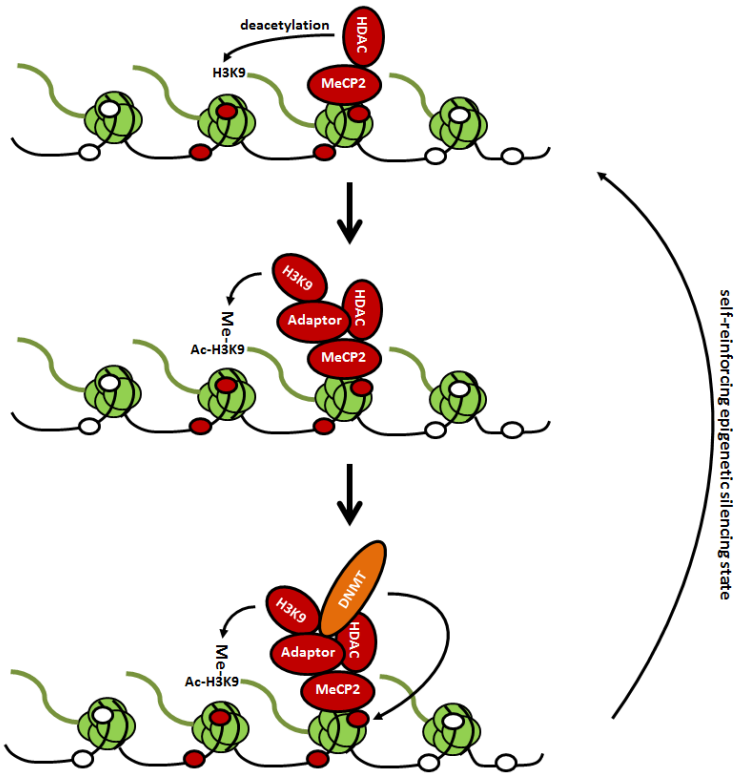


Figure 26. Model for a self-reinforcing epigenetic silencing state to strengthen a repressed chromatin state. CpG is firstly methylated with the help of DNMT and the methylated CpG allows the binding of methyl-CpG-binding domain (MBD) proteins like methyl CpG binding protein 2 (MeCP2) to DNA. Afterwards, histone deacetylase (HDAC) will be recruited to H3K9 and induces H3K9 deacetylation. H3K9 deacetylation would result in histone methylation as deacetylation of H3K9 is necessary for methylation. Furthermore, some specific adaptors will then bind to this repressing complex and recruit again DNMTs to reinforce these silencing procedures.

Combining the model in Figure 25 and model in Figure 26, the data in this thesis implie that not only DNA methylation contribute to the heterogeneous expression pattern of the Tet cassettes in the cells and the mice, but also other histone modifications might play roles in this process. For the cells and mice that did not respond to the treatment, the silencing might have been reinforced

by all the silencing factors all the time and finally achieved the ultimate silencing state; and the responding cells were in plastic state.

4.3. 2 Rescue of the Tet cassette by TET1c-rtTA is reversible

Interestingly, the rescuing of the Tet cassettes by TET1c-rtTA was transient both *in vitro* and *in vivo* (Figure 18-19 and 20-24). For R26-biTetGFP mice the reactivation of luciferase could be detected only for a period of 7 days, while the TET1c-rtTA treated LUC8.10 mice showed a reactivation of luciferase for 21 days. In both cases luciferase expression level dropped to the background levels afterwards. This was also observed in silenced R26-biTetGFP ES cells tranfected with TET1c-rtTA: after one week the reactivation of GFP vanished (data not shown). Of note, re-administration of TET1c-rtTA could re-activate the signal. However, this was also found to be temporal.

This reversibility of reactivation might be the result of the method for applying the plasmid which was achieved by HDTV injection (for mice) and transfection (for cells). The HDTV injection is a highly efficient procedure to deliver nucleic acids to the liver in small animals. By HDTV injection, the plasmid DNA is mainly taken up by the liver and 5-25% hepatocytes could take up the plasmids (Budker et al., 2006). The expression peak of the delivered DNA is usually observed within 12-24 hours, and diminishes after 20-40 hours. This transient expression is attributed to the degradation of the plasmids. Furthermore, less than 1% plasmids has been reported to integrate in the host genome by chance (Crespo et al. 2005; Kovacsics and Raper 2014; Liu et al. 2014; Peng et al. 2015; Suda et al. 2007).

Therefore, the transient reactivation upon injection of TET1c-rtTA by HDTV suggests that the Tet promoter was silenced again as soon as the expression of the TET1c-rtTA declined and the silenced epigenetic state was restored. Thus, a constant expression of TET1c-rtTA would be needed for keeping the Tet promoter in an active state.

Furthermore, this observation might also imply that some repressive mechanisms are ongoing that set in as soon as the demethylated state is not actively maintained.

4.3. 3 Targeted genomic suppression/activation

DNA methylation-induced transgene silencing do not only hampered the antibody production in industry (Spencer et al., 2015) but is also a big problem for transgenesis research (Nair et al., 2011).

In this thesis the catalytic domain of TET1 was fused with the rtTA to engineer a fusion protein TET1c-rtTA for targeted demethylation of the Tet promoter. As shown in Figure 20-24, the engineered TET1c-rtTA fusion has reactivated the Tet cassette in silenced mice and ES cells irrespective of the integration locus.

Targeted demethylation has been shown in 2013 for an endogenous promoter. By combining TET1 hydroxylase catalytic domain with site specific DNA binding motifs targeted demethylation of human endogenous RHOXF2 gene and HBB gene in Hela and HEK293 cells could be achieved (Maeder et al., 2013a). They fused engineered transcription activator–like effector nuclease (TALEN) repeat arrays (targeting either RHOXF2 gene or HBB gene) with the TET1 hydroxylase catalytic domain. Using these TALE-TET1 fusions, they could recruit the demethylation domain to the respective promoter. Thereby they demonstrated that a demethylation of critical promoter CpG positions could be achieved and led to substantial increase in the expression of the targeted endogenous human genes. However, the increase was still transient due to the loss of the plasmids (Maeder et al. 2013a). Soon after that, another study proved that when TET2 was fused to locus guiding Zinc fingers specific for ICAM-1, it successfully reactivated ICAM-1 gene in human ovarian cancer cell line A2780 (Chen et al., 2014).

However, the targeted demethylation in both research studies was very restricted to 1-3 specific CpGs, which might limit the reactivation efficiency. Furthermore, all performed targeted demethylation studies were achieved in cells and not in mice up to now. In this thesis the catalytic domain of TET1 could be recruited to not only one specific position but (due to the 7 repeats) moreover to several sites. This applied method was proved to work not only in cells but also in mice. This suggests that this approach might be more powerful than the published ones and is not limited to *in vitro* studies.

5. OUTLOOK

The results of this study provide proof of principle that efficient reactivation of silenced Tet cassettes can be achieved by targeting the demethylating domain of TET1 to the Tet promoter sequence. This is the first time targeted demethylation of the Tet promoter has been actualized by utilizing TET1. It is predictable that this approach will be a powerful tool to rescue other silenced Tet transgenic mice. This could be achieved by viral transfer of the TET1c-rtTA to the respective cells. As the study pointed out, a constant expression of the TET1c-rtTA is required. To maintain a constant TET1c-rtTA expression in mice, a TET1c-rtTA transgenic mouse strain could be established and crossed to the silenced Tet mice. Thereby, a stable and targeted demethylation would be expected.

The results of this study highlight the complex epigenetic modulation of the Tet cassettes in various sites of the mouse genome. While the study shows that certain strategies for demethylation can improve the expression, a robust activation of particular genomic sites might require a chromosomal context that prevents spreading of negative influences to the transgene cassettes. In this study, it was shown that cHS4 could not fulfill this task. However, other chromatin modifiers, which have been reported to stabilize transgene expression, could be evaluated alone or in combination with the current demethylation strategies. For instance, matrix attachment regions (MARs) or a ubiquitous chromatin opening element (UCOE) (Majocchi et al., 2014; F. Zhang et al., 2010a) could be introduced into the constructs.

In this study, the induced epigenetic modulation was restricted to DNA demethylation. As discussed, other mechanisms might also contribute to silencing and self-reinforcing in particular an active histone repressing machinery. Therefore, to better reactivate the Tet promoter histone modifying enzymatic activities (e.g. histone acetylase or histone methylases) would need to be recruited. For this purpose, the rtTA could be exploited for generating fusion proteins that bind specifically to the Tet promoter. Thus, targeted DNA demethylation and histone modulation could be achieved by the combined administration of both the TET1c-rtTA and histone modulating fusion proteins. It could be envisaged that thereby the stability of the demethylated, active status of the promoter might be increased.

Moreover, this strategy is not restricted to the Tet promoter but could also be utilized for endogenous promoters or any other DNA sequences or histone modification. For instance, with the help of guide RNA from CRISPR/Cas9 system (Hsu et al., 2014) or TALENs (Juillerat et al., 2014), the catalytic domains for DNA demethylation/histone modification could be recruited to the sequences that might 'poison' the locus and induce silencing.

6. REFERENCE

Allshire, R. C., Javerzat, J. P., Redhead, N. J., & Cranston, G. (1994). Position effect variegation at fission yeast centromeres. *Cell*, *76*(1), 157–69.

An, M. C., Zhang, N., Scott, G., Montoro, D., Wittkop, T., Mooney, S., … Ellerby, L. M. (2012). Genetic correction of huntington's disease phenotypes in induced pluripotent stem cells. *Cell Stem Cell*, *11*(2), 253–263. doi:10.1016/j.stem.2012.04.026

Argyros, O., Wong, S. P., Fedonidis, C., Tolmachov, O., Waddington, S. N., Howe, S. J., … Harbottle, R. P. (2011). Development of S/MAR minicircles for enhanced and persistent transgene expression in the mouse liver. *Journal of Molecular Medicine (Berlin, Germany)*, *89*(5), 515–29. doi:10.1007/s00109-010-0713-3

Ashe, A., Morgan, D. K., Whitelaw, N. C., Bruxner, T. J., Vickaryous, N. K., Cox, L. L., … Whitelaw, E. (2008). A genome-wide screen for modifiers of transgene variegation identifies genes with critical roles in development. *Genome Biology*, *9*(12), R182. doi:10.1186/gb-2008-9-12-r182

Baer, A., & Bode, J. (2001). Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Current Opinion in Biotechnology*, *12*(5), 473–480. doi:10.1016/S0958-1669(00)00248-2

Bao-Cutrona, M., & Moral, P. (2009). Unexpected expression pattern of tetracycline-regulated transgenes in mice. *Genetics*, *181*(4), 1687–91. doi:10.1534/genetics.108.097600

Bartkuhn, M., Straub, T., Herold, M., Herrmann, M., Rathke, C., Saumweber, H., … Renkawitz, R. (2009). Active promoters and insulators are marked by the centrosomal protein 190. *The EMBO Journal*, *28*(7), 877–88. doi:10.1038/emboj.2009.34

Bartlett, J. (2010). Increasing Transgene Expression in Barley via the Insertion of an Expression-enhancing Intron, (February), 216–218.

Beard, C., Hochedlinger, K., Plath, K., Wutz, A., & Jaenisch, R. (2006a). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. *Genesis (New York, N.Y. : 2000)*, *44*(1), 23–8. doi:10.1002/gene.20180

Beard, C., Hochedlinger, K., Plath, K., Wutz, A., & Jaenisch, R. (2006b). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. *Genesis (New York, N.Y. : 2000)*, *44*(1), 23–8. doi:10.1002/gene.20180

Bell, A. C., West, A. G., & Felsenfeld, G. (1999). The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell*, *98*(3), 387–96.

Belteki, G., Haigh, J., Kabacs, N., Haigh, K., Sison, K., Costantini, F., … Nagy, A. (2005). Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Research*, *33*(5), e51. doi:10.1093/nar/gni051

Berry, C., Hannenhalli, S., Leipzig, J., & Bushman, F. D. (2006). Selection of target sites for mobile DNA integration in the human genome. *PLoS Computational Biology*, *2*(11), e157. doi:10.1371/journal.pcbi.0020157

Bishop, J. O., & Smith, P. (1989). Mechanism of chromosomal integration of microinjected DNA. *Molecular Biology & Medicine*, *6*(4), 283–98.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248–254. doi:10.1016/0003-2697(76)90527-3

Brooks, A. R., Harkins, R. N., Wang, P., Qian, H. S., Liu, P., & Rubanyi, G. M. (2004). Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *The Journal of Gene Medicine*, *6*(4), 395–404. doi:10.1002/jgm.516

Budker, V. G., Subbotin, V. M., Budker, T., Sebestyén, M. G., Zhang, G., & Wolff, J. A. (2006). Mechanism of plasmid delivery by hydrodynamic tail vein injection. II. Morphological studies. *The Journal of Gene Medicine*, *8*(7), 874–88. doi:10.1002/jgm.920

Burgess-Beusse, B., Farrell, C., Gaszner, M., Litt, M., Mutskov, V., Recillas-Targa, F., … Felsenfeld, G. (2002). The insulation of genes from external enhancers and silencing chromatin. *Proceedings of the National Academy of Sciences of the United States of America*, *99 Suppl 4*, 16433–7. doi:10.1073/pnas.162342499

Cai, H. N., & Shen, P. (2001). Effects of cis arrangement of chromatin insulators on enhancer-blocking activity. *Science (New York, N.Y.)*, *291*(5503), 493–5. doi:10.1126/science.291.5503.493

Carabana, J., Watanabe, A., Hao, B., & Krangel, M. S. (2011). A barrier-type insulator forms a boundary between active and inactive chromatin at the murine TCRβ locus. *Journal of Immunology (Baltimore, Md. : 1950)*, *186*(6), 3556–62. doi:10.4049/jimmunol.1003164

Casola, S. (2010). Mouse models for miRNA expression: the ROSA26 locus. *Methods in Molecular Biology (Clifton, N.J.)*, *667*, 145–63. doi:10.1007/978-1-60761-811-9_10

Chahwan, R., Wontakal, S. N., & Roa, S. (2010). Crosstalk between genetic and epigenetic information through cytosine deamination. *Trends in Genetics : TIG*, *26*(10), 443–8. doi:10.1016/j.tig.2010.07.005

Chen, H., Kazemier, H. G., de Groote, M. L., Ruiters, M. H. J., Xu, G.-L., & Rots, M. G. (2014). Induced DNA demethylation by targeting Ten-Eleven Translocation 2 to the human ICAM-1 promoter. *Nucleic Acids Research*, *42*(3), 1563–74. doi:10.1093/nar/gkt1019

Cheng, A. W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T. W., … Jaenisch, R. (2013). Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Research*, *23*(10), 1163–71. doi:10.1038/cr.2013.122

Choy, M.-K., Movassagh, M., Goh, H.-G., Bennett, M. R., Down, T. A., & Foo, R. S. Y. (2010). Genome-wide conserved consensus transcription factor binding motifs are hyper-methylated. *BMC Genomics*, *11*(1), 519. doi:10.1186/1471-2164-11-519

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., … Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, N.Y.)*, *339*(6121), 819–23. doi:10.1126/science.1231143

Crespo, A., Peydró, A., Dasí, F., Benet, M., Calvete, J. J., Revert, F., & Aliño, S. F. (2005). Hydrodynamic liver gene transfer mechanism involves transient sinusoidal blood stasis and massive hepatocyte endocytic vesicles. *Gene Therapy*, *12*(11), 927–35. doi:10.1038/sj.gt.3302469

Cuddapah, S., Jothi, R., Schones, D. E., Roh, T.-Y., Cui, K., & Zhao, K. (2009). Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. *Genome Research*, *19*(1), 24–32. doi:10.1101/gr.082800.108

Dahl, C., Grønbæk, K., & Guldberg, P. (2011). Advances in DNA methylation: 5-hydroxymethylcytosine revisited. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *412*(11-12), 831–6. doi:10.1016/j.cca.2011.02.013

Davies, S. L., O'Callaghan, P. M., Mcleod, J., Pybus, L. P., Sung, Y. H., Rance, J., … James, D. C. (2011). Impact of gene vector design on the control of recombinant monoclonal antibody production by chinese hamster ovary cells. *Biotechnology Progress*, *27*(6), 1689–1699. doi:10.1002/btpr.692

Dawlaty, M. M., Ganz, K., Powell, B. E., Hu, Y.-C., Markoulaki, S., Cheng, A. W., … Jaenisch, R. (2011). Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. *Cell Stem Cell*, *9*(2), 166–75. doi:10.1016/j.stem.2011.07.010

Dhar, M. K., Vishal, P., Sharma, R., & Kaul, S. (2014). Epigenetic dynamics: role of epimarks and underlying machinery in plants exposed to abiotic stress. *International Journal of Genomics*, *2014*, 187146. doi:10.1155/2014/187146

Doerfler, W. (1992). DNA methylation: eukaryotic defense against the transcription of foreign genes? *Microbial Pathogenesis*, *12*(1), 1–8.

Ed, F. R. (2000). Inducible Gene Expression and Gene Modification in Transgenic Mice, 95–100.

Ellis, J. (2005). Silencing and variegation of gammaretrovirus and lentivirus vectors. *Human Gene Therapy*, *16*(11), 1241–6. doi:10.1089/hum.2005.16.1241

Emery, D. W. (2011). The use of chromatin insulators to improve the expression and safety of integrating gene transfer vectors. *Human Gene Therapy*, *22*(6), 761–74. doi:10.1089/hum.2010.233

Emery, D. W., Yannaki, E., Tubb, J., & Stamatoyannopoulos, G. (2000). A chromatin insulator protects retrovirus vectors from chromosomal position effects. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(16), 9150–5. doi:10.1073/pnas.160159597

Everett, R. S., Evans, H. K., Hodges, B. L., Ding, E. Y., Serra, D. M., & Amalfitano, a. (2004). Strain-specific rate of shutdown of CMV enhancer activity in murine liver confirmed by use of persistent [E1(-), E2b(-)] adenoviral vectors. *Virology*, *325*(1), 96–105. doi:10.1016/j.virol.2004.04.032

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K., & Sander, J. D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology*, *31*(9), 822–6. doi:10.1038/nbt.2623

Fuks, F. (2005). DNA methylation and histone modifications: teaming up to silence genes. *Current Opinion in Genetics & Development*, *15*(5), 490–5. doi:10.1016/j.gde.2005.08.002

Fuks, F., Hurd, P. J., Wolf, D., Nan, X., Bird, A. P., & Kouzarides, T. (2003). The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *The Journal of Biological Chemistry*, *278*(6), 4035–40. doi:10.1074/jbc.M210256200

Gaál, Z., & Oláh, E. (2014). [Epigenetic regulatory mechanisms and their disorders in leukemia]. *Magyar Onkologia*, *58*(2), 99–107.

Garrick, D., Fiering, S., Martin, D. I., & Whitelaw, E. (1998). Repeat-induced gene silencing in mammals. *Nature Genetics*, *18*(1), 56–9. doi:10.1038/ng0198-56

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(39), E2579–86. doi:10.1073/pnas.1208507109

Globisch, D., Münzel, M., Müller, M., Michalakis, S., Wagner, M., Koch, S., … Carell, T. (2010). Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. *PloS One*, *5*(12), e15367. doi:10.1371/journal.pone.0015367

Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A., & Ruddle, F. H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *77*(12), 7380–4.

Grajevskaja, V., Balciuniene, J., & Balciunas, D. (2013). Chicken β-globin insulators fail to shield the nkx2.5 promoter from integration site effects in zebrafish. *Molecular Genetics and Genomics : MGG*, *288*(12), 717–25. doi:10.1007/s00438-013-0778-0

Gu, T.-P., Guo, F., Yang, H., Wu, H.-P., Xu, G.-F., Liu, W., … Xu, G.-L. (2011). The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature*, *477*(7366), 606–10. doi:10.1038/nature10443

Guelen, L., Pagie, L., Brasset, E., Meuleman, W., Faza, M. B., Talhout, W., … van Steensel, B. (2008). Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. *Nature*, *453*(7197), 948–51. doi:10.1038/nature06947

Guo, J. U., Su, Y., Zhong, C., Ming, G., & Song, H. (2011a). Emerging roles of TET proteins and 5-hydroxymethylcytosines in active DNA demethylation and beyond. *Cell Cycle (Georgetown, Tex.)*, *10*(16), 2662–8.

Guo, J. U., Su, Y., Zhong, C., Ming, G., & Song, H. (2011b). Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell*, *145*(3), 423–34. doi:10.1016/j.cell.2011.03.022

Gupta, S., Kim, S. Y., Artis, S., Molfese, D. L., Schumacher, A., Sweatt, J. D., … Lubin, F. D. (2010). Histone methylation regulates memory formation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *30*(10), 3589–99. doi:10.1523/JNEUROSCI.3732-09.2010

Haenebalcke, L., Goossens, S., Naessens, M., Kruse, N., Farhang Ghahremani, M., Bartunkova, S., … Haigh, J. J. (2013). Efficient ROSA26-based conditional and/or inducible transgenesis using RMCE-compatible F1 hybrid mouse embryonic stem cells. *Stem Cell Reviews*, *9*(6), 774–85. doi:10.1007/s12015-013-9458-z

He, Y.-F., Li, B.-Z., Li, Z., Liu, P., Wang, Y., Tang, Q., … Xu, G.-L. (2011). Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science (New York, N.Y.)*, *333*(6047), 1303–7. doi:10.1126/science.1210944

Herold, M., Bartkuhn, M., & Renkawitz, R. (2012). CTCF: insights into insulator function during development. *Development (Cambridge, England)*, *139*(6), 1045–57. doi:10.1242/dev.065268

Hillen, W., & Berens, C. (1994). EXPRESSION OF TNIO ENCODED TETRACYCLINE RESISTANCE Wolfgang Hillen and Christian Berens.

Holohan, E. E., Kwong, C., Adryan, B., Bartkuhn, M., Herold, M., Renkawitz, R., … White, R. (2007). CTCF genomic binding sites in Drosophila and the organisation of the bithorax complex. *PLoS Genetics*, *3*(7), e112. doi:10.1371/journal.pgen.0030112

Hou, C., Dale, R., & Dean, A. (2010). Cell type specificity of chromatin organization mediated by CTCF and cohesin. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(8), 3651–6. doi:10.1073/pnas.0912087107

Howden, B. P., Beaume, M., Harrison, P. F., Hernandez, D., Schrenzel, J., Seemann, T., … Stinear, T. P. (2013). Analysis of the small RNA transcriptional response in multidrug-resistant Staphylococcus aureus after antimicrobial exposure. *Antimicrobial Agents and Chemotherapy*, *57*(8), 3864–74. doi:10.1128/AAC.00263-13

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell*, *157*(6), 1262–1278. doi:10.1016/j.cell.2014.05.010

Ito, S., D'Alessio, A. C., Taranova, O. V, Hong, K., Sowers, L. C., & Zhang, Y. (2010). Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*, *466*(7310), 1129–33. doi:10.1038/nature09303

Ito, S., Shen, L., Dai, Q., Wu, S. C., Collins, L. B., Swenberg, J. A., … Zhang, Y. (2011). Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science (New York, N.Y.)*, *333*(6047), 1300–3. doi:10.1126/science.1210597

Jabbari, K., & Bernardi, G. (2004). Cytosine methylation and CpG, TpG (CpA) and TpA frequencies. *Gene*, *333*, 143–9. doi:10.1016/j.gene.2004.02.043

Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics*, *33 Suppl*, 245–54. doi:10.1038/ng1089

Juillerat, A., Dubois, G., Valton, J., Thomas, S., Stella, S., Maréchal, A., … Duchateau, P. (2014). Comprehensive analysis of the specificity of transcription activator-like effector nucleases. *Nucleic Acids Research*, *42*(8), 5390–402. doi:10.1093/nar/gku155

Jüttermann, R., Li, E., & Jaenisch, R. (1994). Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(25), 11797–801.

Kanduri, C., Pant, V., Loukinov, D., Pugacheva, E., Qi, C. F., Wolffe, A., … Lobanenkov, V. V. (2000). Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive. *Current Biology : CB*, *10*(14), 853–6.

Kang, S. H., Kwon, J. Y., Lee, J. K., & Seo, Y. R. (2013). Recent advances in in vivo genotoxicity testing: prediction of carcinogenic potential using comet and micronucleus assay in animal models. *Journal of Cancer Prevention*, *18*(4), 277–88.

Kantarjian, H., Issa, J.-P. J., Rosenfeld, C. S., Bennett, J. M., Albitar, M., DiPersio, J., … Saba, H. (2006). Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer*, *106*(8), 1794–803. doi:10.1002/cncr.21792

Kong, Q., Wu, M., Huan, Y., Zhang, L., Liu, H., Bou, G., … Liu, Z. (2009). Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs. *PloS One*, *4*(8), e6679. doi:10.1371/journal.pone.0006679

Kovacsics, D., & Raper, J. (2014). Transient expression of proteins by hydrodynamic gene delivery in mice. *Journal of Visualized Experiments : JoVE*, (87), e51481. doi:10.3791/51481

Krestel, H. E., Shimshek, D. R., Jensen, V., Nevian, T., Kim, J., Geng, Y., … Seeburg, P. H. (2004). A genetic switch for epilepsy in adult mice. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *24*(46), 10568–78. doi:10.1523/JNEUROSCI.4579-03.2004

Kriaucionis, S., & Heintz, N. (2009). The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science (New York, N.Y.)*, *324*(5929), 929–30. doi:10.1126/science.1169786

Kues, W. A., Schwinzer, R., Wirth, D., Verhoeyen, E., Lemme, E., Herrmann, D., … Niemann, H. (2006). Epigenetic silencing and tissue independent expression of a novel tetracycline inducible system in double-transgenic pigs. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *20*(8), 1200–2. doi:10.1096/fj.05-5415fje

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., & Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, *410*(6824), 116–20. doi:10.1038/35065132

Lehnertz, B., Ueda, Y., Derijck, A. A. H. A., Braunschweig, U., Perez-Burgos, L., Kubicek, S., … Peters, A. H. F. M. (2003). Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Current Biology : CB*, *13*(14), 1192–200.

Li, F., Vijayasankaran, N., Shen, A. (Yijuan), Kiss, R., & Amanullah, A. (2014). Cell culture processes for monoclonal antibody production. *mAbs*, *2*(5), 466–479. doi:10.4161/mabs.2.5.12720

Li, T., Hu, J.-F., Qiu, X., Ling, J., Chen, H., Wang, S., … Hoffman, A. R. (2008). CTCF regulates allelic expression of Igf2 by orchestrating a promoter-polycomb repressive complex 2 intrachromosomal loop. *Molecular and Cellular Biology*, *28*(20), 6473–82. doi:10.1128/MCB.00204-08

Li, T., Lu, Z., & Lu, L. (2004). Regulation of eye development by transcription control of CCCTC binding factor (CTCF). *The Journal of Biological Chemistry*, *279*(26), 27575–83. doi:10.1074/jbc.M313942200

Li, X., Ito, M., Zhou, F., Youngson, N., Zuo, X., Leder, P., & Ferguson-Smith, A. C. (2008). A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Developmental Cell*, *15*(4), 547–57. doi:10.1016/j.devcel.2008.08.014

Ling, J. Q., Li, T., Hu, J. F., Vu, T. H., Chen, H. L., Qiu, X. W., … Hoffman, A. R. (2006). CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. *Science (New York, N.Y.)*, *312*(5771), 269–72. doi:10.1126/science.1123191

Litt, M. D., Simpson, M., Recillas-Targa, F., Prioleau, M. N., & Felsenfeld, G. (2001). Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *The EMBO Journal*, *20*(9), 2224–35. doi:10.1093/emboj/20.9.2224

Liu, Z., Gan, L., Yang, X., Zhang, Z., & Sun, C. (2014). Hydrodynamic tail vein injection of SOCS3 eukaryotic expression vector in vivo promoted liver lipid metabolism and hepatocyte apoptosis in mouse. *Biochemistry and Cell Biology = Biochimie et Biologie Cellulaire*, *92*(2), 119–25. doi:10.1139/bcb-2013-0117

Livingstone, B. (2012). EPIGENETICS, Linking Genotype and Phenotype in Development and Evolution edited by B.Hallgrímsson, and B. K.Hall, eds. Berkeley and Los Angeles ; University of California Press, 2011. 457pp. Hardcover. ISBN 978-0-520-26709-1. £59. *Biological Journal of the Linnean Society*, *107*(4), 953–953. doi:10.1111/j.1095-8312.2012.01949.x

Loew, R., Heinz, N., Hampf, M., Bujard, H., & Gossen, M. (2010). Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnology*, *10*, 81. doi:10.1186/1472-6750-10-81

Lorsbach, R. B., Moore, J., Mathew, S., Raimondi, S. C., Mukatira, S. T., & Downing, J. R. (2003). TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). *Leukemia*, *17*(3), 637–41. doi:10.1038/sj.leu.2402834

Lübbert, M. (2000). DNA methylation inhibitors in the treatment of leukemias, myelodysplastic syndromes and hemoglobinopathies: clinical results and possible mechanisms of action. *Current Topics in Microbiology and Immunology*, *249*, 135–64.

Maeder, M. L., Angstman, J. F., Richardson, M. E., Linder, S. J., Cascio, V. M., Tsai, S. Q., … Joung, J. K. (2013a). Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. *Nature Biotechnology*, *31*(12), 1137–42. doi:10.1038/nbt.2726

Maeder, M. L., Angstman, J. F., Richardson, M. E., Linder, S. J., Cascio, V. M., Tsai, S. Q., … Joung, J. K. (2013b). Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. *Nature Biotechnology*, *31*(12), 1137–42. doi:10.1038/nbt.2726

Maeder, M. L., Thibodeau-Beganny, S., Osiak, A., Wright, D. A., Anthony, R. M., Eichtinger, M., … Joung, J. K. (2008). Rapid “open-source” engineering of customized zinc-finger nucleases for highly efficient gene modification. *Molecular Cell*, *31*(2), 294–301. doi:10.1016/j.molcel.2008.06.016

Majocchi, S., Aritonovska, E., & Mermod, N. (2014). Epigenetic regulatory elements associate with specific histone modifications to prevent silencing of telomeric genes. *Nucleic Acids Research*, *42*(1), 193–204. doi:10.1093/nar/gkt880

Mattick, J. S., Amaral, P. P., Dinger, M. E., Mercer, T. R., & Mehler, M. F. (2009). RNA regulation of epigenetic processes. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology*, *31*(1), 51–9. doi:10.1002/bies.080099

Matzke, M. A., & Mette, M. F. (2000). Transgene silencing by the host genome defense : implications for the evolution of epigenetic control mechanisms in plants and vertebrates, 401–415.

Meehan, R. R., Lewis, J. D., McKay, S., Kleiner, E. L., & Bird, A. P. (1989). Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. *Cell*, *58*(3), 499–507. doi:10.1016/0092-8674(89)90430-3

Meyer, P. (2013). Transgenes and their contributions to epigenetic research. *The International Journal of Developmental Biology*, *57*(6-8), 509–15. doi:10.1387/ijdb.120254pm

Morita, E., Arii, J., Christensen, D., Votteler, J., & Sundquist, W. I. (2012). Attenuated protein expression vectors for use in siRNA rescue experiments. *BioTechniques*, 1–5. doi:10.2144/000113909

Nair, A. R., Jinger, X., & Hermiston, T. W. (2011). Effect of different UCOE-promoter combinations in creation of engineered cell lines for the production of Factor VIII. *BMC Research Notes*, *4*(1), 178. doi:10.1186/1756-0500-4-178

Nan, X., Meehan, R. R., & Bird, A. (1993). Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Research*, *21*(21), 4886–4892.

Ndlovu, M. N., Denis, H., & Fuks, F. (2011). Exposing the DNA methylome iceberg. *Trends in Biochemical Sciences*, *36*(7), 381–7. doi:10.1016/j.tibs.2011.03.002

Nehlsen, K., da Gama-Norton, L., Schucht, R., Hauser, H., & Wirth, D. (2011). Towards rational engineering of cells: Recombinant gene expression in defined chromosomal loci. *BMC Proceedings*, *5 Suppl 8*, O6. doi:10.1186/1753-6561-5-S8-O6

Newell-Price, J., Clark, A. J., & King, P. (n.d.). DNA methylation and silencing of gene expression. *Trends in Endocrinology and Metabolism: TEM*, *11*(4), 142–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10754536

Ohlsson, R., Bartkuhn, M., & Renkawitz, R. (2010). CTCF shapes chromatin by multiple mechanisms: the impact of 20 years of CTCF research on understanding the workings of chromatin. *Chromosoma*, *119*(4), 351–60. doi:10.1007/s00412-010-0262-0

Olivares-Villagómez, D., Algood, H. M. S., Singh, K., Parekh, V. V, Ryan, K. E., Piazuelo, M. B., … Van Kaer, L. (2011). Intestinal epithelial cells modulate CD4 T cell responses via the thymus leukemia antigen. *Journal of Immunology (Baltimore, Md. : 1950)*, *187*(8), 4051–60. doi:10.4049/jimmunol.1101052

Oyer, J. a, Chu, A., Brar, S., & Turker, M. S. (2009). Aberrant epigenetic silencing is triggered by a transient reduction in gene expression. *PloS One*, *4*(3), e4832. doi:10.1371/journal.pone.0004832

Pai, A., Bell, J., Marioni, J., Pritchard, J., & Gilad, Y. (2011). A genome-wide study of {DNA} methylation patterns and gene expression levels in multiple human and chimpanzee tissues. *{PLoS} Genetics*, *7*(2), e1001316. doi:10.1371/journal.pgen.1001316

Palais, G., Nguyen Dinh Cat, A., Friedman, H., Panek-Huet, N., Millet, A., Tronche, F., … Jaisser, F. (2009). Targeted transgenesis at the HPRT locus: an efficient strategy to achieve tightly

controlled in vivo conditional expression with the tet system. *Physiological Genomics*, *37*(2), 140–6. doi:10.1152/physiolgenomics.90328.2008

Pankiewicz, R., Karlen, Y., Imhof, M. O., & Mermod, N. (2005). Reversal of the silencing of tetracycline-controlled genes requires the coordinate action of distinctly acting transcription factors. *The Journal of Gene Medicine*, *7*(1), 117–32. doi:10.1002/jgm.644

Papapetrou, E. P., Lee, G., Malani, N., Setty, M., Riviere, I., Tirunagari, L. M. S., … Sadelain, M. (2011). Genomic safe harbors permit high β-globin transgene expression in thalassemia induced pluripotent stem cells. *Nature Biotechnology*, *29*(1), 73–8. doi:10.1038/nbt.1717

Pedram, M., Sprung, C. N., Gao, Q., Lo, A. W. I., Reynolds, G. E., & Murnane, J. P. (2006). Telomere position effect and silencing of transgenes near telomeres in the mouse. *Molecular and Cellular Biology*, *26*(5), 1865–78. doi:10.1128/MCB.26.5.1865-1878.2006

Peng, X.-H., Ren, X.-N., Chen, L.-X., Shi, B.-S., Xu, C.-H., Fang, Z., … Zhou, X.-H. (2015). High persistence rate of hepatitis B virus in a hydrodynamic injection-based transfection model in C3H/HeN mice. *World Journal of Gastroenterology : WJG*, *21*(12), 3527–36. doi:10.3748/wjg.v21.i12.3527

Persons, D. A., Hargrove, P. W., Allay, E. R., Hanawa, H., & Nienhuis, A. W. (2003). The degree of phenotypic correction of murine beta -thalassemia intermedia following lentiviral-mediated transfer of a human gamma-globin gene is influenced by chromosomal position effects and vector copy number. *Blood*, *101*(6), 2175–83. doi:10.1182/blood-2002-07-2211

Piccolo, F. M., Bagci, H., Brown, K. E., Landeira, D., Soza-Ried, J., Feytout, A., … Fisher, A. G. (2013). Different roles for Tet1 and Tet2 proteins in reprogramming-mediated erasure of imprints induced by EGC fusion. *Molecular Cell*, *49*(6), 1023–33. doi:10.1016/j.molcel.2013.01.032

Pichlmair, A., Kandasamy, K., Alvisi, G., Mulhern, O., Sacco, R., Habjan, M., … Superti-Furga, G. (2012). Viral immune modulators perturb the human molecular network by common and unique strategies. *Nature*, *487*(7408), 486–490. doi:10.1038/nature11289

Premsrirut, P. K., Dow, L. E., Kim, S. Y., Camiolo, M., Malone, C. D., Miething, C., … Lowe, S. W. (2011). A rapid and scalable system for studying gene function in mice using conditional RNA interference. *Cell*, *145*(1), 145–58. doi:10.1016/j.cell.2011.03.012

Prockop, D. J., & Kivirikko, K. I. (1995). Collagens: molecular biology, diseases, and potentials for therapy. *Annual Review of Biochemistry*, *64*, 403–34. doi:10.1146/annurev.bi.64.070195.002155

Ptashne, M. (2007). On the use of the word “epigenetic”. *Current Biology : CB*, *17*(7), R233–6. doi:10.1016/j.cub.2007.02.030

Quilici, L. S., Silva-Pereira, I., Andrade, a. C., Albuquerque, F. C., Brigido, M. M., & Maranhão, a. Q. (2013). A minimal cytomegalovirus intron A variant can improve transgene expression in different mammalian cell lines. *Biotechnology Letters*, *35*(1), 21–27. doi:10.1007/s10529-012-1043-z

Raab, J. R., & Kamakaka, R. T. (2010). Insulators and promoters: closer than we think. *Nature Reviews. Genetics*, *11*(6), 439–46. doi:10.1038/nrg2765

Recillas-Targa, F., Pikaart, M. J., Burgess-Beusse, B., Bell, A. C., Litt, M. D., West, A. G., … Felsenfeld, G. (2002). Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(10), 6883–8. doi:10.1073/pnas.102179399

Rincón-Arano, H., Furlan-Magaril, M., & Recillas-Targa, F. (2007). Protection against telomeric position effects by the chicken cHS4 beta-globin insulator. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(35), 14044–9. doi:10.1073/pnas.0704999104

Rubio, E. D., Reiss, D. J., Welcsh, P. L., Disteche, C. M., Filippova, G. N., Baliga, N. S., … Krumm, A. (2008). CTCF physically links cohesin to chromatin. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(24), 8309–14. doi:10.1073/pnas.0801273105

Sandhu, U., Cebula, M., Behme, S., Riemer, P., Wodarczyk, C., Metzger, D., … Wirth, D. (2011). Strict control of transgene expression in a mouse model for sensitive biological applications based on RMCE compatible ES cells. *Nucleic Acids Research*, *39*(1), e1. doi:10.1093/nar/gkq868

Sanyal, A., Lajoie, B. R., Jain, G., & Dekker, J. (2012). The long-range interaction landscape of gene promoters. *Nature*, *489*(7414), 109–13. doi:10.1038/nature11279

Sapp, J. (1991). Concepts of organization. The leverage of ciliate protozoa. *Developmental Biology (New York, N.Y. : 1985)*, *7*, 229–58. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1804215

Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., & Siksnys, V. (2011). The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Research*, *39*(21), 9275–82. doi:10.1093/nar/gkr606

Sharma, N., Hollensen, A. K., Bak, R. O., Staunstrup, N. H., Schrøder, L. D., & Mikkelsen, J. G. (2012). The impact of cHS4 insulators on DNA transposon vector mobilization and silencing in retinal pigment epithelium cells. *PloS One*, *7*(10), e48421. doi:10.1371/journal.pone.0048421

Shi, Q., Tian, X., Zhao, Y., Luo, H., Tian, Y., & Luo, A. (2014). Anti-arthritic effects of FasL gene transferred intra-articularly by an inducible lentiviral vector containing improved tet-on system. *Rheumatology International*, *34*(1), 51–7. doi:10.1007/s00296-011-2002-z

Shockett, P. E., & Schatz, D. G. (1996). Commentary Diverse strategies for tetracycline-regulated inducible gene expression, *93*(May), 5173–5176.

Singer, S. D., Liu, Z., & Cox, K. D. (2012). Minimizing the unpredictability of transgene expression in plants: the role of genetic insulators. *Plant Cell Reports*, *31*(1), 13–25. doi:10.1007/s00299-011-1167-y

Sinn, P. L., Burnight, E. R., Hickey, M. A., Gary, W., & Mccray, P. B. (2005). Persistent Gene Expression in Mouse Nasal Epithelia Following Baculovirus GP64 Pseudotyped FIV-Based Gene Transfer Effects of the cHS4 Insulator Elements on Efficient Gene Transfer Using GALV-Pseudotyped, *11*(May), 5–6.

Song, C.-X., Szulwach, K. E., Fu, Y., Dai, Q., Yi, C., Li, X., … He, C. (2011). Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nature Biotechnology*, *29*(1), 68–72. doi:10.1038/nbt.1732

Song, N., Liu, J., An, S., Nishino, T., Hishikawa, Y., & Koji, T. (2011). Immunohistochemical Analysis of Histone H3 Modifications in Germ Cells during Mouse Spermatogenesis. *Acta Histochemica et Cytochemica*, *44*(4), 183–90. doi:10.1267/ahc.11027

Spencer, S., Gugliotta, A., Koenitzer, J., Hauser, H., & Wirth, D. (2015). Stability of single copy transgene expression in CHOK1 cells is affected by histone modifications but not by DNA methylation. *Journal of Biotechnology*, *195*, 15–29. doi:10.1016/j.jbiotec.2014.12.009

Stadtfeld, M., Maherali, N., Borkent, M., & Hochedlinger, K. (2010). A reprogrammable mouse strain from gene-targeted embryonic stem cells. *Nature Methods*, *7*(1), 53–5. doi:10.1038/nmeth.1409

Stieger, K., Belbellaa, B., Le Guiner, C., Moullier, P., & Rolling, F. (2009). In vivo gene regulation using tetracycline-regulatable systems. *Advanced Drug Delivery Reviews*, *61*(7-8), 527–41. doi:10.1016/j.addr.2008.12.016

Stresemann, C., & Lyko, F. (2008). Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *International Journal of Cancer. Journal International Du Cancer*, *123*(1), 8–13. doi:10.1002/ijc.23607

Suda, T., Gao, X., Stolz, D. B., & Liu, D. (2007). Structural impact of hydrodynamic injection on mouse liver. *Gene Therapy*, *14*(2), 129–37. doi:10.1038/sj.gt.3302865

Sui, X., Price, C., Li, Z., & Chen, J. (2012). Crosstalk Between DNA and Histones : Tet ' s New Role in Embryonic Stem Cells, 603–608.

Sun, Y., Chen, X., & Xiao, D. (2007). Tetracycline-inducible Expression Systems: New Strategies and Practices in the Transgenic Mouse Modeling. *Acta Biochimica et Biophysica Sinica*, *39*(4), 235–246. doi:10.1111/j.1745-7270.2007.00258.x

Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. a, Bandukwala, H., Brudno, Y., … Rao, A. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science (New York, N.Y.)*, *324*(5929), 930–5. doi:10.1126/science.1170116

Takahashi, Y., Watanabe, T., Nakagawa, S., Kawakami, K., & Sato, Y. (2008). Transposon-mediated stable integration and tetracycline-inducible expression of electroporated transgenes in chicken embryos. *Methods in Cell Biology*, *87*, 271–80. doi:10.1016/S0091-679X(08)00214-8

Takiguchi, M., Dow, L. E., Prier, J. E., Carmichael, C. L., Kile, B. T., Turner, S. J., … Dickins, R. a. (2013). Variability of inducible expression across the hematopoietic system of tetracycline transactivator transgenic mice. *PloS One*, *8*(1), e54009. doi:10.1371/journal.pone.0054009

Tchorz, J. S., Suply, T., Ksiazek, I., Giachino, C., Cloëtta, D., Danzer, C. P., … Mueller, M. (2012a). A modified RMCE-compatible Rosa26 locus for the expression of transgenes from exogenous promoters. *PLoS ONE*, *7*(1).

Tchorz, J. S., Suply, T., Ksiazek, I., Giachino, C., Cloëtta, D., Danzer, C.-P., … Mueller, M. (2012b). A modified RMCE-compatible Rosa26 locus for the expression of transgenes from exogenous promoters. *PloS One*, *7*(1), e30011. doi:10.1371/journal.pone.0030011

Thesis Kruse. (2013). *Establishment of a mouse model for liver specific HCV expression*.

Thesis Spence. (2014). *Crosstalk of synthetic cassettes in defined chromosomal sites*.

Togo, S., Sato, T., Sugiura, H., Wang, X., Basma, H., Nelson, A., … Rennard, S. I. (2011). Differentiation of embryonic stem cells into fibroblast-like cells in three-dimensional type I collagen gel cultures. *In Vitro Cellular & Developmental Biology. Animal*, *47*(2), 114–24. doi:10.1007/s11626-010-9367-2

Uchida, N., Hanawa, H., Yamamoto, M., & Shimada, T. (2013). The chicken hypersensitivity site 4 core insulator blocks promoter interference in lentiviral vectors. *Human Gene Therapy Methods*, *24*(2), 117–24. doi:10.1089/hgtb.2012.152

Uchida, S., Sakai, S., Furuichi, T., Hosoda, H., Toyota, K., Ishii, T., … Kida, S. (2006). Tight regulation of transgene expression by tetracycline-dependent activator and repressor in brain. *Genes, Brain, and Behavior*, *5*(1), 96–106. doi:10.1111/j.1601-183X.2005.00139.x

Wan, M., Gu, H., Wang, J., Huang, H., Zhao, J., Kaundal, R. K., … Chi, T. (2013). Inducible mouse models illuminate parameters influencing epigenetic inheritance. *Development (Cambridge, England)*, *140*(4), 843–52. doi:10.1242/dev.088229

Wang, P., Qu, J., Wu, M.-Z., Zhang, W., Liu, G.-H., & Izpisua Belmonte, J. C. (2013). "TET-on" pluripotency. *Cell Research*, *23*(7), 863–5. doi:10.1038/cr.2013.72

West, A. G., Gaszner, M., & Felsenfeld, G. (2002). Insulators: many functions, many mechanisms. *Genes & Development*, *16*(3), 271–88. doi:10.1101/gad.954702

West, A. G., Huang, S., Gaszner, M., Litt, M. D., & Felsenfeld, G. (2004). Recruitment of histone modifications by USF proteins at a vertebrate barrier element. *Molecular Cell*, *16*(3), 453–63. doi:10.1016/j.molcel.2004.10.005

Williams, K., Christensen, J., Pedersen, M. T., Johansen, J. V, Cloos, P. a C., Rappsilber, J., & Helin, K. (2011). TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature*, *473*(7347), 343–8. doi:10.1038/nature10066

Wörtge, S., Eshkind, L., Cabezas-Wallscheid, N., Lakaye, B., Kim, J., Heck, R., … Bockamp, E. (2010a). Tetracycline-controlled transgene activation using the ROSA26-iM2-GFP knock-in mouse strain permits GFP monitoring of DOX-regulated transgene-expression. *BMC Developmental Biology*, *10*, 95. doi:10.1186/1471-213X-10-95

Wörtge, S., Eshkind, L., Cabezas-Wallscheid, N., Lakaye, B., Kim, J., Heck, R., … Bockamp, E. (2010b). Tetracycline-controlled transgene activation using the ROSA26-iM2-GFP knock-in mouse strain permits GFP monitoring of DOX-regulated transgene-expression. *BMC Developmental Biology*, *10*(1), 95. doi:10.1186/1471-213X-10-95

Wu, H., & Zhang, Y. (2011). Mechanisms and functions of Tet protein- mediated 5-methylcytosine oxidation, 2436–2452. doi:10.1101/gad.179184.111.genomes

Xue, W., Chen, S., Yin, H., Tammela, T., Papagiannakopoulos, T., Joshi, N. S., … Jacks, T. (2014). CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature*, *514*(7522), 380–384. doi:10.1038/nature13589

Yao, S., Osborne, C. S., Bharadwaj, R. R., Pasceri, P., Sukonnik, T., Pannell, D., … Ellis, J. (2003). Retrovirus silencer blocking by the cHS4 insulator is CTCF independent. *Nucleic Acids Research*, *31*(18), 5317–23.

Yu, C., Zhang, Y.-L., Pan, W.-W., Li, X.-M., Wang, Z.-W., Ge, Z.-J., … Fan, H.-Y. (2013). CRL4 complex regulates mammalian oocyte survival and reprogramming by activation of TET proteins. *Science (New York, N.Y.)*, *342*(6165), 1518–21. doi:10.1126/science.1244587

Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G., & Soriano, P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(8), 3789–94.

Zeng, H., Horie, K., Madisen, L., Pavlova, M. N., Gragerova, G., Rohde, A. D., … Gaitanaris, G. a. (2008). An inducible and reversible mouse genetic rescue system. *PLoS Genetics*, *4*(5), e1000069. doi:10.1371/journal.pgen.1000069

Zhang, C., Huys, A., Thibault, P. a., & Wilson, J. a. (2012). Requirements for human Dicer and TRBP in microRNA-122 regulation of HCV translation and RNA abundance. *Virology*, *433*(2), 479–488. doi:10.1016/j.virol.2012.08.039

Zhang, F., Frost, A. R., Blundell, M. P., Bales, O., Antoniou, M. N., & Thrasher, A. J. (2010a). A ubiquitous chromatin opening element (UCOE) confers resistance to DNA methylation-mediated silencing of lentiviral vectors. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *18*(9), 1640–9. doi:10.1038/mt.2010.132

Zhang, F., Frost, A. R., Blundell, M. P., Bales, O., Antoniou, M. N., & Thrasher, A. J. (2010b). A ubiquitous chromatin opening element (UCOE) confers resistance to DNA methylation-mediated silencing of lentiviral vectors. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *18*(9), 1640–1649. doi:10.1038/mt.2010.132

Zhang, H., Zhang, X., Clark, E., Mulcahey, M., Huang, S., & Shi, Y. G. (2010). TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. *Cell Research*, *20*(12), 1390–3. doi:10.1038/cr.2010.156

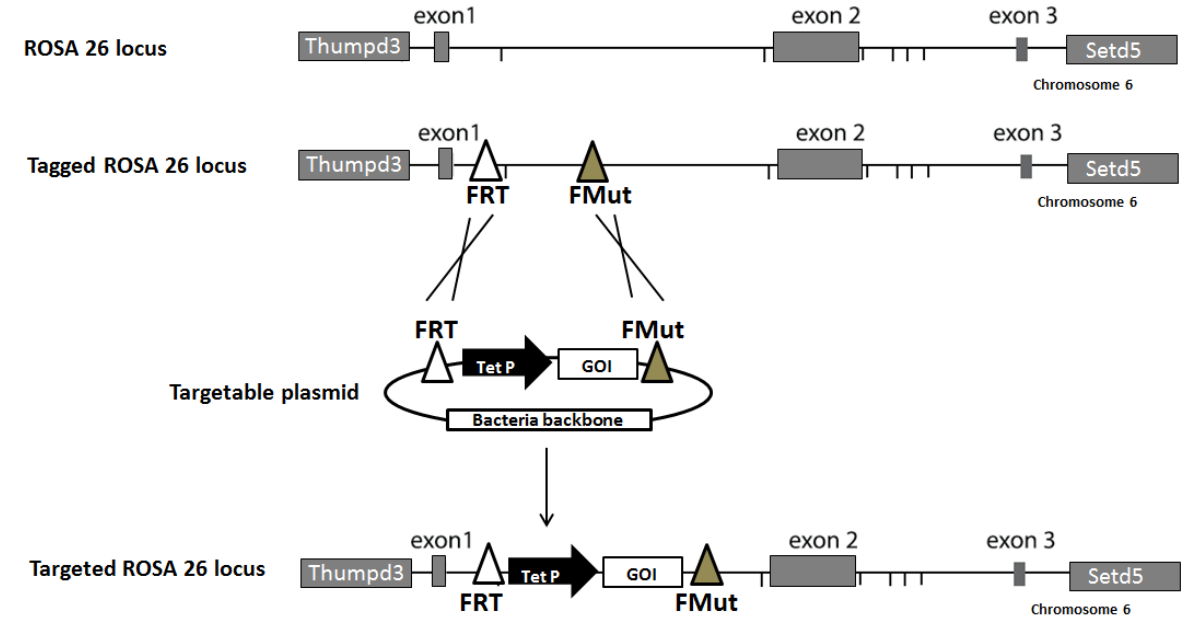
Zhu, P., Aller, M. I., Baron, U., Cambridge, S., Bausen, M., Herb, J., … Hasan, M. T. (2007a). Silencing and un-silencing of tetracycline-controlled genes in neurons. *PloS One*, *2*(6), e533. doi:10.1371/journal.pone.0000533

Zhu, P., Aller, M. I., Baron, U., Cambridge, S., Bausen, M., Herb, J., … Hasan, M. T. (2007b). Silencing and un-silencing of tetracycline-controlled genes in neurons. *PloS One*, *2*(6), e533. doi:10.1371/journal.pone.0000533

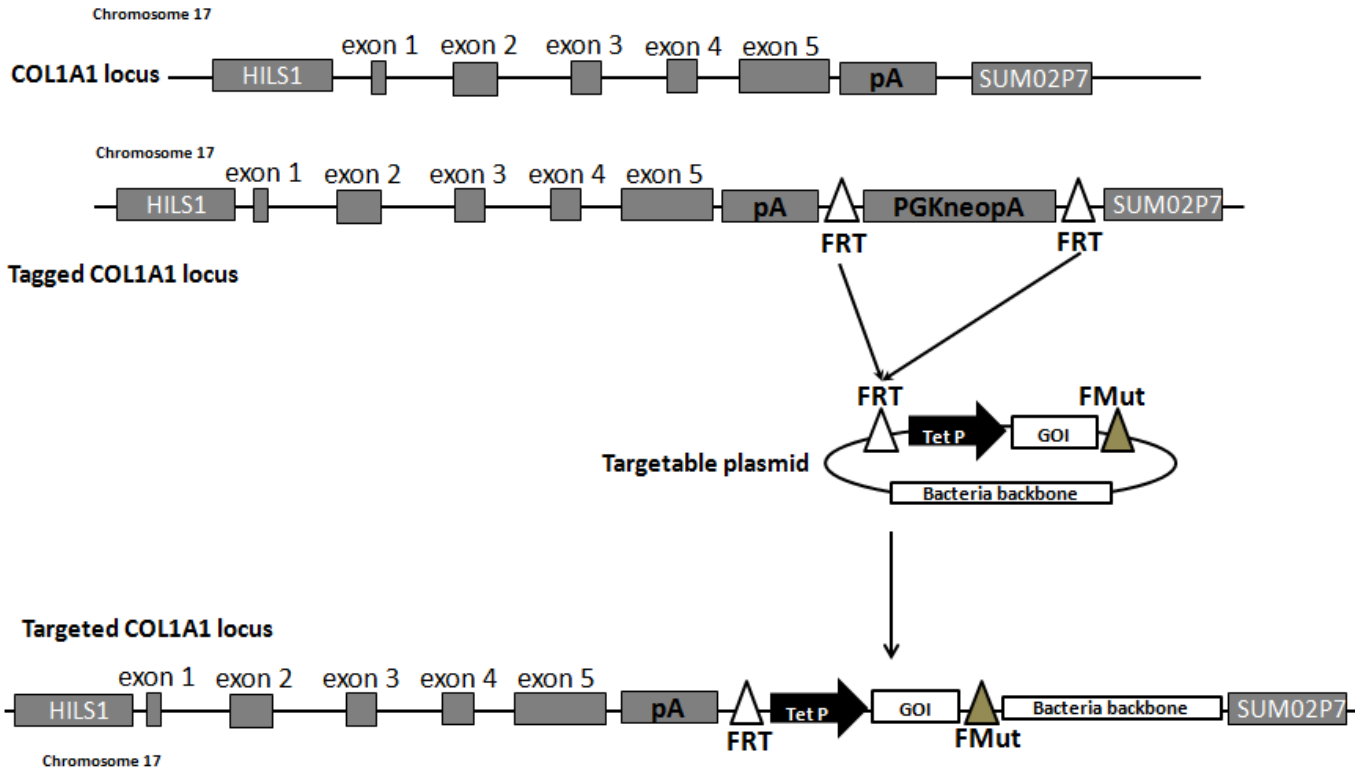
7. APPENDIX

7.1 Tagged ES cells and targeting strategy

a.)



b.)



c.)

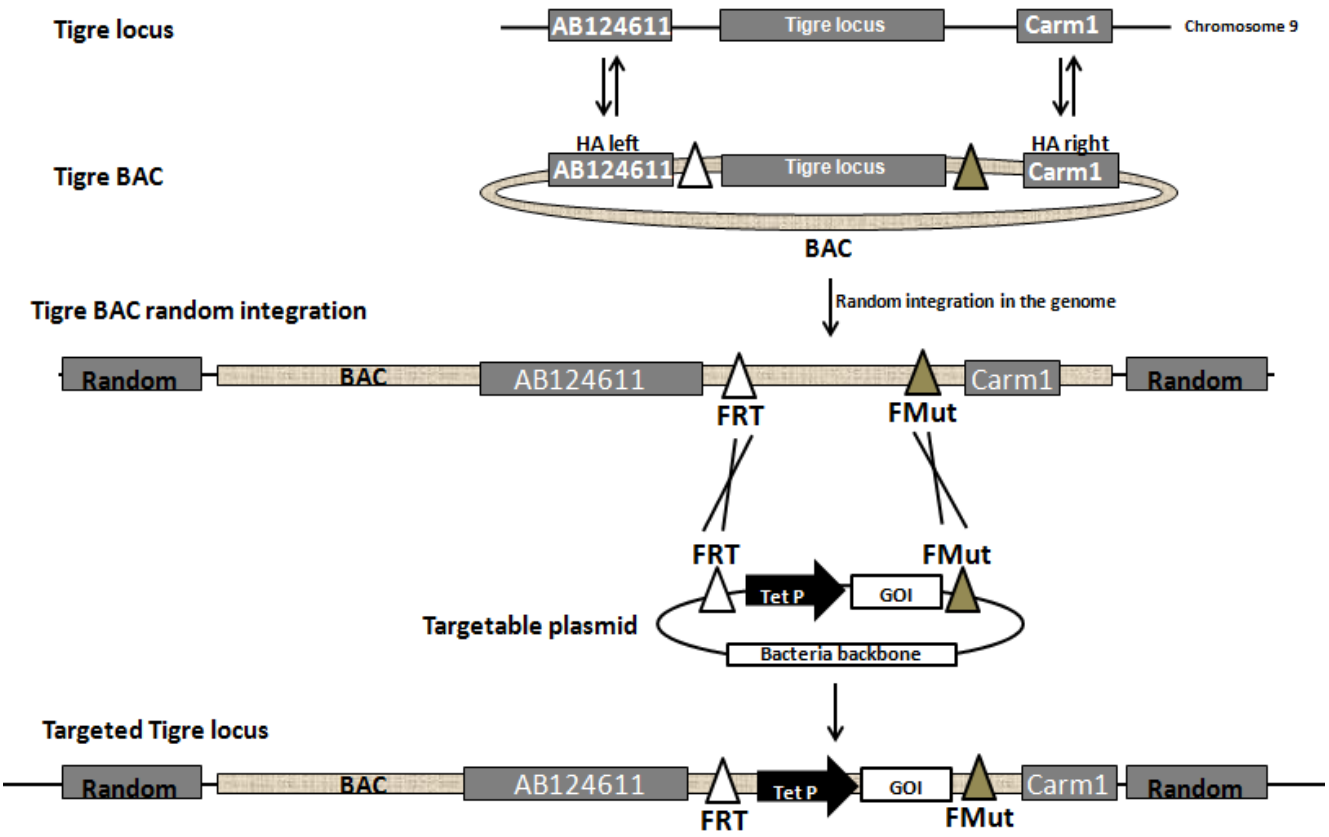


Figure S1: FLP dependent targeting strategies for different loci: a.) The Rosa26 locus lies on chromosome 6 in between the setd5 gene and Thumpd3 gene. It has been tagged by heterospecific FRT sites (FRT wt and FRT Mutant) after the first exon (RosaAntiluc/G4B12 ES cells) (Sandhu et al., 2011). The targeting cassette carries the same heterospecific FRT sites. The Tetracycline inducible cassettes are flanked by such heterogeneous FRT sites (targetable plasmid) allowing the recombinase (FLP) mediated cassette exchange (RMCE) into the tagged locus. b.) The COL1A1 locus lies on chromosome 17 in between the HILS1 gene and SUMO02P7 gene and has been tagged after the fifth exons (Beard et al.,). The tagged COL1A1 locus carries two FRT wt sites. In between a PGK promoter driving neo resistance and poly A signal is placed. Thus, upon targeting the full plasmid will be integrated into the locus including the bacteria backbone, while the PGKneopA will be deleted c.) The Tigre locus is located on chromosome 9 in between of the AB124611 gene and Carm1 gene. A Bacterial Artificial Chromose (BAC) vector comprising this locus was modified with FRT and FRTmut sites in vitro and randomly transferred to ES cells. A clone carrying a single copy of this BAC was isolated (Wirth, et al., not published). For targeting, a cassette flanked by the same FRT and FRTmut sites is introduced in Tigre locus upon FLP treatment.

7.2 Abbreviation

5'HS4	constitutive DNase I-hypersensitive site
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosin
5mC	5-methylcytosine
alcA	alcohol dehydrogenase I
AML	acute myeloid leukemia
ANK1	Ankyrin 1
AZA	5-azacytidine
BER	base excision repair
CCL4	Carbon Tetrachloride
CGBP	CpG-binding protein
ChIP	Chromatin Immunoprecipitation
cHS4	Chicken β-globin insulator
CL	clone
CMV	cytomegalovirus promoter
COL1A1	type I collagen locus
CRISPR-Cas	Targeted transgenesis based on cas9
	DNA nuclease enzyme mediated DNA double-strand breaks
CTCF	CCCTC-binding factors
CXXC domain	CXXC-type zinc finger protein binding domain

DEC	Decitabine
DME	Demeter
DNMT	DNA methyl-transferases
dNTP	deoxyribonucleotide triphosphate
DOX	Doxcycline
DSBs	double-strand breaks
E.coli	Escherichia coli
EB	embryoid bodies
ER	estrogen receptor
ES	embryonic stem cells
FACS	Fluorescence-activated cell sorting
FCS	fetal calf serum
Fibro-L	fibroblast like cells
FR	folate receptor
FRT	flippase recognition target sites
GFP	green fluorescent protein
GOI	gene of interest
GR	glucocorticoid receptor
GSH	Genomic safe harbor
H11	Hipp11
HAT	histone acetyltransferase
HCV	Hepatitis C Virus
HDAC	Histone deacetylase

HDTV	Hydro dynamic tail veil injection
Hep-L	Hepatic-like
HR	homologous recombination
ICR	imprinting control region
JFH1	subgenomic replicon of HCV
kb	1000 base(s)
KO	knock out
LHFPL4	Lipoma HMGIC Fusion Partner-Like 4 Protein
MBPs	methyl binding proteins
MeCP2	methyl CpG binding protein 2
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MLL	mixed-lineage leukemia
mRNA	messenger RNA
MTMR14	Myotubularin-related protein 14
NHEJ	non-homologous end-joining
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEV	position effect variegation
PI	pronuclear injection
PTetbi	Bi-directional Doxycycline (Tetracycline) inducible promoter
RIGS	repeat-induced gene silencing
RMCE	recombinase mediate cassette exchange

ROS1	repressor of silencing 1
Rosa26	gene trap ROSA 26 locus
rtTA	reverse tetracycline transactivator
SAM	S-adenosyl-L-methionine
SETD5	SET Domain-Containing Protein 5
sgRNA	guide RNA
SRGAP3	SLIT-ROBO Rho GTPase activating protein 3
TAF	transcription initiation factor
TALENs	transcription activator-like effector nucleases
TBP	TATA-binding protein
Tet	Tetracycline (doxycycline) inducible expression cassette
Tet promoter	Doxycycline (Tetracycline) inducible promoter
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TET1c	Catalytic domain from TET1 protein
TET1c-rtTA	Catalytic domain from TET1 protein fused with rtTA
TET1c-unspecific	Catalytic domain from TET1 without binding domain
Tet-O	bacterial Tet-operator
Tet-Off	tTA dependent inducible expression system
Tet-On	rtTA dependent inducible expression system
TetR	Tetracycline repressor protein
THUMPD3	THUMP Domain-Containing Protein 3
TIigre	tightly regulated locus
tracr RNA	trans-activating CRISPR RNA

Tris	tris hydroxymethylaminomethane
tTA	TetR+VP16
UCRs	ultra-conserved regions
USFs	upstream stimulatory factors
UV	ultraviolet
VC	Ascorbic acid
Vezf1	vascular endothelial zinc finger 1
VPA	valproic acid
ZF	zinc finger nucleases

7.3 list of figures

Figure26: Model for a self-reinforcing epigenetic silencing state to strengthen a repressed chromatin state.

7.4 list of tables